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THE PHILOSOPHY OF THE EVALUATION OF DISINFECTANTS AND ANTISEPTICS

BY G. SYKES

Address of the President of the Society for Applied Bacteriology, delivered to the meeting of the Society on Tuesday, 24 October, 1961

INTRODUCTION

WHEN ROBERT KOCH in 1881 produced his silk thread technique for testing for disinfectant activity he probably did not realize how much in advance of his time he was; much less could he appreciate what comparatively little progress would be made during the next eighty years or so, or what an endless stream of problems he had engendered for those engaged in this field of applied bacteriology. If one examines the evolution of the subject since Koch's time one finds that it falls into three phases, the first being signalled by the introduction of the Rideal-Walker and Chick-Martin tests in 1903 and 1908 respectively and thereby laying the basis for all subsequent phenol coefficient, and the frequently so-called 'suspension' tests. The second occurred in the middle 1930s, with a revival of interest in tests with bacteria dried on surfaces, or 'surface film' tests, and the third ran parallel with the other two and involved studies on the dynamics of disinfection, as initiated by Kronig and Paul in 1897, and led to statistical appraisals of end-points and other relevant factors.

This line of evolution has been concerned mainly with disinfectants, or preparations used for treating inanimate materials, but alongside have been developed methods for assessing the efficiencies of antiseptics, or preparations intended for application to the skin and the various body tissues and membranes. The latter have been developed under a much less intense aura of publicity and are perhaps all the better for it. They differ from the disinfectant-type tests in one significant aspect, namely, they are more objective and in general have been designed each with a specific application in mind. I shall have more to say on this later.

EVALUATING AND TESTING DISINFECTANTS

In considering methods for determining disinfectant activity one must be quite clear about one's terms of reference and the purpose of the determination: whether it is simply to check the quality of routine production material or whether it is to assess the performance characteristics of a preparation, for these two approaches are quite different. The former is concerned only with finding out whether the preparation conforms to a given germicidal standard under a fixed set of conditions—conditions which, as in the Rideal-Walker and similar coefficient tests, are quite artificial in many respects—whereas evaluation implies determining whether it is efficacious in practice for the purposes required, that is, whether it remains active under a variety of natural conditions, against a varied range of organisms with possible differences in temperature, in periods of contact, in the concentrations and types of organic matter present, and so on.

This does not mean that there is an unbridged, or unbridgeable, gulf between the two approaches: neither should it imply that the phenol coefficient test is too unrealistic to be of any value in practice. Indeed, if used intelligently such a test can provide useful information for routine control purposes; it is only when the test is misapplied that difficulties arise.

The two great errors perpetuated in the use of phenol coefficient tests over the years have been their improper and indiscriminate application to almost any type of antibacterial preparation, when they were only consciously devised by their originators for testing the coal-tar, emulsion-type disinfectants, and the repeated attempts, in spite of protests raised as early as 1909, to evaluate a disinfectant or antiseptic on the basis of a single test. I am glad to say that after much toil both of these are now being put in their right perspective.

We must not, of course, denigrate these earlier tests too much. They were, in fact, based on a reasoning which at the time appeared to be sound, but which in the light of subsequent knowledge has unfortunately been found wanting. Koch, for instance, used anthrax spores in his test because he knew them to be highly resistant and at the same time realized their epidemiological significance. Similarly, Rideal and Walker chose *Salmonella typhi*, or the old *Bacterium typhosum*, as their test organism because the disinfectants they were examining were used only for general sanitary purposes; therefore an organism of the typhoid-colon group was an obvious choice and *Salm. typhi* was thought to be more suitable than, say, *Escherichia coli*. They also deliberately chose a short disinfection time of about 5 minutes because, to use Rideal's words, "any lengthening of the time of the test . . . is open to the objection that we want disinfectants to act in the shortest possible time", and they chose phenol, quite naturally, as the reference standard because they were only concerned with phenolic germicides and this was the well established and well tried antiseptic since Lister's day. Again, Chick and Martin introduced dried faeces into their test in an attempt to make the conditions more realistic; likewise several later workers have used organisms dried in various ways on surfaces because often this more nearly represents the practical conditions in which a disinfectant is required to act.

It has been said in more than one quarter that it is "the big financial interests" of the disinfectant industry which are responsible for retaining the now outmoded Rideal-Walker type test, and that without such support the test would die of disuse. But this, I am certain, is a gross exaggeration of the facts. No one is wedded to, or even in love with, the Rideal-Walker test and so no one has any particular desire to see it or its progeny perpetuated. I am equally certain that industry, if presented with a more reproducible and more informative test, would be only too pleased to adopt it, even for routine control purposes. In fact I believe we have the structure of such a test in a recent British Standard Specification for the laboratory evaluation of the disinfectant activity of quaternary ammonium compounds. Although designed primarily around the quaternaries, it can be applied to all types of disinfectants and it has the particular advantage that within the structure the detailed conditions can be varied according to the intended uses of the disinfectant.

Even so, we have still only made a *laboratory* evaluation of the preparation. The conditions in the test may have been adjusted to simulate as nearly as possible those in which it is likely to be used in practice and from this we can hope that the results will be similar. But we cannot be certain of this and it cannot be over emphasized that only field trials will give the real answer. Here, however, we are confronted with other problems. Such trials are often difficult to plan with any degree of certainty, or to execute in such a manner that the results can be assessed satisfactorily to the exclusion of other interfering factors. Thus we often have to make the best use of the resources and facilities available and trust that the laboratory evidence so obtained will give the desired result when applied in practice.

DEFECTS IN EXISTING TESTS

Much has been said and written on the defects and faults in the existing methods for assessing disinfectants and I do not wish to labour this point unduly. Nevertheless, some points need to be reiterated if only to remind ourselves that these problems are still with us unsolved. They include the choice of an optimum time of contact of the disinfectant with the organism (which will inevitably vary according to the proposed use of the disinfectant), the choice of a suitable and repeatable end-point in terms of rate or amount of kill, the selection of appropriate test organisms, the establishment of satisfactory reference standards and the choice of temperature both in the test itself and for the cultivation of the test organisms.

Temperature. The present official methods all specify rather close temperature ranges within which the test must be carried out, that of the Rideal-Walker being the lowest at 17-18°. Here again there may have been logical reasons at the time for making such choices, but from evidences now available a greater degree of flexibility could be allowed without unduly affecting the result. There is still, of course, a good argument for assessing activity for general use at normal ambient temperatures, and also at a higher temperature approaching 37° for certain specific purposes, but the exact temperature need not be as rigidly specified as was at first supposed.

Of far greater significance, I am certain, is the way in which the organisms are handled before and immediately after the actual disinfection treatment. For instance, no one has yet told us what is the effect on the resistance and similar characteristics of cultures grown at temperatures only slightly removed from their optimum. We all know that our laboratory incubators, although set nominally at, say, 37°, inevitably may vary by a degree or so, and even worse may fluctuate, and the effect of such variations on the organisms might well be profound. There is a need to investigate this point and probably a good case for growing any test organism at a temperature significantly below its normal optimum.

A similar argument can be applied to the recovery of treated organisms. It has always been naively assumed that because an organism under normal conditions grows best at a certain temperature, then this temperature must be the optimum under all conditions. But this is not always the case, and we have increasing evidence to this effect. We know, for example, that after exposure to ultraviolet or gamma radiation and probably after heat treatment, damaged cells will recover better at temperatures substantially below their normal optimum. The same might well apply in chemical disinfection, but so far this question does not seem to have received any attention.

In passing one might draw attention to a little noticed defect in the technique for assessing the survival of organisms by plate counts. Before a culture can be used in a test it must first be grown for a period, usually in an incubator at 37° for 24 hours. It is then cooled in a water bath to the temperature of the test and subjected to a major assault by the disinfectant. After this the injured cells are diluted in broth, saline or some other fluid, probably at a temperature slightly different from that of the test. Then they are heated practically instantaneously to 40° or so when inoculated into the molten agar, after which they are allowed to cool while the agar sets; lastly they are transferred to their final resting place in the incubator, again at 37°. No wonder few of them survive! Here is a case for using a liquid culture medium where the post-disinfection treatment is at least a little more kind to the damaged cells.

Reference standards. Discussions on the choice of suitable reference standards for disinfectants are likely to continue for some years to come. Traditionally phenol has always been used but in the light of present knowledge a more unfortunate choice probably could not have been made. It is a water soluble compound of small molecular size, whereas many of the preparations it is intended to control contain higher and more complex phenols along with a wide range of emulsifying or solubilizing agents. On these grounds alone one might expect them to behave differently and to possess entirely different bactericidal characteristics, and such is the case. Their dilution coefficients are different and they even show differing day-to-day fluctuations in their actual killing concentrations.

Such an argument inevitably leads to the suggestion that each type of disinfectant, if not each individual formulation, should have its own standard, but here there are difficulties particularly when dealing with the emulsion-type disinfectants. However, if we return to an earlier theme of this discourse and think in terms of an evaluation—still keeping the method as simple as possible—is there any need for a standard at all? May it not be feasible to rely on a direct performance without reference to any other material? There are those, in fact, who have already suggested using phenol in the present tests only to make sure that the organism is 'behaving properly' and not employing it as a means of obtaining a coefficient. This could be an immediate and simple step out of the valley of fixity and merits further consideration.

Choice of organisms. Hitherto most of the techniques described, with the notable exceptions of the American F.D.A. and A.O.A.C. methods, have specified only one test organism, but I am quite certain that this is entirely inadequate for assessing the practical efficacy of any disinfectant. What, therefore, is to be the range of choice? The field is wide open, but fortunately many of the possibilities are either self selected or self eliminated. Thus, we are only interested in those infective organisms which are relatively highly resistant and persistent—any treatment which will deal with these will automatically deal with the less resistant ones. Similarly, there is general agreement that the sporeforming bacteria should not be included because most disinfectants in common use are not very effective against such organisms; hence the treatment of these highly resistant types tends to be considered as a special case.

In the past, disinfection has always been linked with sanitation in a general sense and so organisms of the intestinal group have been the natural choice, the coli-aerogenes organisms and salmonellae being particular favourites. Often selected strains of *E. coli* have been chosen, because besides being indicators of faecal contamination they were known to be robust and not at all exacting in their nutritional requirements. On this account they were thought to be less sensitive to small variations in cultural conditions, hence more reproducible in terms of their resistance and recovery characteristics. Experience, however, shows *E. coli* to be just as variable as some others of the Gram-negative group, and so there is no reason to restrict the choice on these grounds.

Latterly the emphasis has moved from the intestinal contaminants to the Gram-positive cocci, particularly the staphylococci, which have loomed so large in recent years in hospital cross-infections, and the pseudomonads, especially *Pseudomonas pyocyanea*, which is so highly resistant and still a source of trouble in wound infections. On these pre-eminently practical grounds, therefore, these two organisms should be included in any selection and, in fact, they are probably adequate in themselves for making any assessment or evaluation.

The necessity for using more than one test organism arises from the nature of the disinfectants themselves. With the refinements in formulation now taking place, the incorporation of new or modified antibacterial substances and the introduction of different types of solubilizers and carriers, there is a tendency for some types of disinfectant to become somewhat selective in their activities and be more effective against the Gram-positive than the Gram-negative organisms, or *vice versa*. In some cases, for example, there may be a comparatively enhanced activity against *Salm. typhi* and formulations can be devised simply for the purpose of boosting the Rideal-Walker coefficient—but the activity may be much lower against *Staphylococcus aureus*. The Americans have gone some distance in controlling this by specifying in their official phenol coefficient tests the use of both *Salm. typhi* and *Staph. aureus*, with a third organism, *Salm. choleraesuis*, in their 'Use-Dilution' test. In this country similar steps have been taken by introducing a staphylococcal coefficient test complementary to the Rideal-Walker test.

Finally, one last point on this topic: various workers have suggested using several different strains of any selected species in order to ensure a better spread of resistance. Desirable as this may seem, it is somewhat idealistic and impracticable, because however many strains may be chosen, even though recently isolated, one cannot guarantee that the most resistant one has been included. The best that can be done, therefore, is to choose a strain which is reasonably representative of the type and refer back frequently to a freeze-dried stock to avoid attenuation due to prolonged continuous subculture.

Choice of end-point. The end-point in any disinfectant test can be considered from two distinct, though somewhat interlocking, points of view: the killing time, or time during which the disinfectant can be allowed to act, and the percentage or level of kill required. Because we are dealing with bacterial infections, ideally one would like to see a total kill of the organisms concerned. Tradition has it that if viable cells are not found in a standard loopful of the disinfectant–culture mixture then this desirable objective has been achieved, but we have known for a long time, in fact since the end of the last century, that the death of a bacterial population under any form of treatment is never an instantaneous but always a gradual and progressive process. Hence there cannot be a sudden and sharply defined transition from a culture with viable to one with nonviable cells. Nevertheless it is only comparatively recently that more detailed investigations, and particularly the application of statistics, have revealed how wrong and misleading such a presumption can be.

When a bacterial system is in a dynamic state, such as occurs during a disinfection process, and the cells are losing viability at a controlled rate, a stage will be reached where the numbers of cells surviving are such that there is *on the average* less than one/loopful. A single loopful, therefore, may or may not contain a viable cell and on this hangs whether the subculture shows growth or no growth, that is, whether at this point the result is positive or negative. Thus the presumed 100% kill is not a real end-point but a random hit-or-miss affair depending exclusively on the chance transfer of a single survivor unit, and this applies whether the original inoculum is a light one or is counted in millions. Increasing the volume of liquid subcultured beyond that of the normal loopful does not materially alter the situation, but only shifts the zone of indecision. Thus one can see the fallacy of the single growth–no growth end-point as practised in the present official tests, and if further evidence is required it is only necessary to turn to the statistician who can prove, in the case of the American F.D.A. test for instance, that within the conditions specified normal variations in the phenol coefficient value of up to 360% are to be expected.

Yet another facet of this fallacy has only comparatively recently come to light. The end-point in all of the present tests has long been recognized to depend not on the average resistance but on the most resistant cells of a culture, but there seems to be increasing evidence that such cells may be abnormal. In some very recent published work, for instance, these almost lone survivor cells, under some circumstances, have been shown to be able to reverse the disinfection process and, by virtue of the exudates of their sister cells which have already been killed, actually start to grow again and produce progeny approaching numerically the original inoculum. Evidently these resistant cells possess an unusual degree of adaptability and for this reason may be thought to be abnormal in relation to the culture as a whole.

This discovery might also account for another phenomenon which is often encountered in following a disinfection process. The survivor-time curve is generally accepted to follow a logarithmic or sigmoidal form, but this may not always be the case. Recently, for instance, we have observed that the initial death-rate of a culture can be quite rapid up to about a 98–99% kill, after which it quickly falls off and the remainder of the time-survivor curve becomes relatively flat and considerably extended. This might well be the phenomenon of adaptation showing itself, although the disinfectant contact time in this case is admittedly rather short for such an occurrence. It could also help to explain the phenomenon of indeterminate end-points and why, as we have found in some cases, the survivor rate appears unexpectedly to be on a numerical basis rather than being a function of the size of the original inoculum.

Incidentally, we know that certain of the properties of micro-organisms can be disturbed by adverse conditions, but I have been unable to find any references to changes in the relative infectivities of organisms consequent on a disinfection treatment.

Having virtually rejected the pseudo-100% kill as an end-point, where should this point now be fixed? In nearly all other biological assays a 50% response is used, but in disinfectant testing this seems incongruous and the reasons for this are several. First, in dealing with the large numbers of individual cells used in this type of work it only adds to the complexity of the test to attempt to assess the point of such a high survival rate; second, a reduction in a bacterial population of only 50% is of no practical significance; third, the dilution coefficients of different types of preparation can vary so greatly that a result at this level can be misleading; fourth, and probably most significant, the rate of kill at this point is near the maximum and is therefore subject to the greatest experimental errors. We are thus left with a choice lying somewhere between 50% and 100% kill with the bias strongly towards the 100% level. From all considerations, a reasonable choice would be a kill of 99·9% or greater and in my opinion this should be the aim in any future test design.

Turning to the second aspect of the subject, namely, the contact time during which such an end-point can be achieved, it always appears evident to me that a 5 or 10 minute period such as is used in the present tests is much too short. During this time the state of viability of the inoculum is changing at such a rapid rate that within the limitations of techniques available the errors must inevitably be great. In this respect the Chick-Martin method, in which a 30 minute killing time is used, is much to be preferred. During this longer period the mortality curve is considerably lengthened and this enables the end-point to be found more reliably within the critical zone on the curve. There seems to be no advantage in prolonging the period beyond 30 minutes.

Evaluating Antiseptics

When we come to consider the evaluation of antiseptic preparations in contrast to disinfectants the situation, as I have already indicated, is quite different and seems much more susceptible to reason. It is realized that if one wants to use an antiseptic to disinfect the skin or to treat an infection in the mouth there is no virtue or value in knowing its Rideal-Walker or similar value. Thus, no longer are we tied to a phenol equivalent response at a fixed time, temperature and so on, and there is a greater flexibility, each type of test being much more nearly related to the actual conditions of usage.

If the preparation to be examined is an antiseptic ointment or cream there are at least two types of test which can be employed. In the first a measure of the release of the medicament from its base and its penetrability is obtained by means of an agar diffusion technique using a variety of organisms. In the second the lethal time of the preparation is obtained by overlaying it on an agar surface culture of the test organism and cutting out discs at intervals to assess the survivors. Both are valuable tests in determining the practical efficacy of preparations designed for topical applications.

Similarly, in dealing with skin disinfectants, including the so-called antiseptic soaps, a range of tests can be applied such as the hand basin washing techniques and direct skin swabbing tests: mouse tail methods have also been proposed, but even though they allow pathogenic organisms to be used they have never been popular and are now superseded. The principle of the hand basin washing technique, of which there are several variations, is that the hands of test subjects are treated with the antiseptic preparation, after which they are rinsed in a succession of known volumes of sterile water, some of which are subsequently cultured quantitatively to enumerate the remaining viable bacteria. By comparison with control subjects the efficacy of the treatment in terms of percentage reduction of the viable bacteria can be assessed. The great difficulty and drawback with this, as with other tests using human subjects, is that because of the differences between the skin bacterial populations of individuals, and even in the same individual at different times, large numbers of replicates, or test subjects, are required to obtain reproducible and meaningful results.

In the skin swabbing tests relatively small areas of the skin on the forearm or the internodes of the fingers are treated with the antiseptic and then swabbed at intervals. This type of test is self contained in that control areas on the same subject can be used: also it allows more than one time interval, dilution of antiseptic, etc. to be compared. But its greatest value is that it can be used for measuring retention of activity on the skin. For this purpose selected areas of the skin are treated with the antiseptic and then, after a lapse of say two or three hours a suitable test organism is applied to the same area. After a few minutes' contact the areas are swabbed and the survivors enumerated. Such a test can be used, for example, to demonstrate the efficacy of the chloroxylenol preparations.

In testing oral antiseptics, including sweets and lozenges, one is again up against the extreme variability of the bacterial content of the mouth, both in types and in numbers. The method usually involves dissolving or otherwise introducing the antiseptic in the mouth, and then taking a series of mouth rinses. I have never had any success with this method and have had to resort to *in vitro* evaluations in which the preparation is dissolved or diluted in saliva and the killing time at 37° of an inoculum of a streptococcus or a staphylococcus determined. Because such preparations must be relatively quick acting, the killing times are usually measured in seconds and minutes.

The foregoing are all tests of a rather special nature, but besides these there are several fairly standard tests which can be applied as appropriate to antiseptics in general. These include tests to determine the bacteriostatic and bactericidal characteristics of the preparation and are usually made in water or nutrient broth. With antiseptic preparations, however, it is important to know these values in the presence of serum and possibly other body fluids and the tests must be adjusted accordingly: in the case of wound antiseptics whole blood should be used.

PROGNOSIS FOR DISINFECTANT EVALUATION

And now, what of the future? In terms of antiseptic evaluation, apart from a certain desirable rationalization of some of the methods, there seems to be little difficulty. But with disinfectants the situation is quite different, and in this context let me make three categorical statements:

1. There is no future for the phenol coefficient type of test.
2. An entirely fresh and untrammelled approach to the problem is needed.
3. In the meantime there is probably little point in trying to 'improve' the existing tests. Such improvements as can be envisaged are unlikely to reconcile the divergent results often found between laboratories, although there is always the possibility that they might expose the reasons for them.

I think I have said enough in the earlier part of my address to indicate some of the points needing consideration in any new technique, although the picture is far from complete. In terms of the organisms to be used in such a technique, clearly at least two different types are needed, and *Staph. aureus* and *Ps. pyocyanea* seem to be adequate and most appropriate for the purpose. Objections may be raised against the latter on account of its exceptional robustness and therefore one of the coli-aerogenes organisms might also be included. The temperatures at which such organisms are cultivated in preparation for the test and recovered after the disinfection treatment are subjects requiring further investigation.

In terms of a suitable contact time between organism and disinfectant there will always be specific cases where quite short disinfection times are needed, but for general purposes 30 minutes seems to be a reasonable optimum; 15 minutes or less is too short on account of the rapid rate of kill and periods beyond half an hour seem unduly long.

One of the major points needing re-appraisal is the definition and determination of the end-point. Various attempts have been made to circumvent the present traditional methods, including enzyme inactivation and nephelometric procedures as well as other novel ideas based on the correlation of some property of the cell with its viability, but all with little success. The most promising seems to be by a replicate subculture technique in which, instead of relying on a single, maybe adventitious, plus or minus response a number of responses are obtained, some positive and some negative, thus giving a more precise assessment. This can be achieved either by replicate subcultures of small volumes direct from the medication mixture in the usual way or by transferring a single larger amount to a relatively large volume of a suitable diluting or quenching fluid from which replicate small volumes are subsequently cultured. The latter is the procedure of choice because by adjusting the amounts of the diluting fluid and medication mixture transferred, etc., any predetermined level of kill in the desired range of 99·9% or greater can be covered.

Finally, there remain to be discussed two questions which have not yet been mentioned. The first concerns the use of different surfaces in the tests and of organisms dried on them, and the second the inclusion of organic and suspended matter. Only rarely is a disinfectant required to be used in a plain aqueous medium, hence the inclusion of organic matter of some sort seems inevitable and fundamental; likewise, much disinfection work involves treating organisms on various soiled surfaces ranging from glassware and milk churns to blankets and linen, and all of these conditions need to be borne in mind for any ultimate evaluation. With antiseptics such considerations are almost automatic, but not so with disinfectants. We have, of course, the Chick-Martin technique which specifies the use of organic matter in the form of killed yeast cells, and the results obtained from such a test only serve to underline its importance in practical testing. Similarly we have the A.O.A.C. 'Use-Dilution' test, which employs a test organism dried on small steel cylinders, and there are several other tests mainly directed towards dairy disinfection which use glass, metal and rubber surfaces with a variety of 'soiling' materials.

When the Chick-Martin technique was first produced it included dried human faeces as the organic matter, but later experience showed that yeast cells could equally well be used. The exact choice of the type of organic matter may therefore not be important, although some differentiation should probably be made between suspended and dissolved matter. In practice the materials most likely to be encountered are milk, blood or serum, and faecal and urinary matter, and in most cases they will be self selected in any testing regimen.

On the question of the introduction of 'surface film' in contrast to 'suspension' type tests the situation is rather more difficult. What kind of surface, for instance, and what sort of drying menstruum should be used? Obviously a disinfectant will behave differently on a hard, impervious surface such as glass or metal than it will on rubber, and even more differently on a fibrous or porous surface such as wood or textile. Moreover, the film in which the organisms are dried might by its nature be expected to exert a greater or less degree of protection against attack by the disinfectant, and for this reason some workers have suggested using a standard soiling mixture consisting of varying amounts of raw egg, whole milk, butter and other fats.

There are those, of course, who have stated that no advantage is to be gained from complicating an already difficult test in this way, using the arguments (a) that the wall of the tube or container in which the disinfection is carried out itself provides an adequate surface, and (b) that having wetted a dried film the organisms therein are released into the disinfectant solution and so are no more protected than are those suspended in a liquid containing organic matter. On the other hand there are those who are convinced that such assumptions can be misleading, particularly at present when the disinfectants available are so diverse in their constitution and formulation, and consequently in their physical characteristics including surface activity and detergency. These properties are intrinsic to the efficacy of a disinfectant but they are largely masked in a purely liquid suspension test, hence the need for direct tests with the appropriate surfaces. This argument applies particularly to porous surfaces and fibrous material, especially wool and cotton goods, which are known to require higher concentrations of all disinfectants than do metal or glass surfaces.

Obviously there is no point in doing surface film tests before a disinfectant has been evaluated by suspension-type tests. They should, in fact, constitute the last phase in any evaluation programme.

CONCLUSION

In summary, therefore, "When we are able to say that the temperature at which disinfection is to be carried out will modify the necessary strength of the dilution by multiplying or dividing by a given figure for each degree above or below that taken as the standard; when we are able to refer to similar figures to modify our coefficient, representing each type of organism to be destroyed, the type of surrounding conditions in which it is found, the type of material to be disinfected and its surface, soft, hard, irregular, or spongy, that soak up fluids or repel them and the like: when we are able to modify the figures according to the physical and chemical nature—emulsion or solution—of the diluted disinfectant, to allow for the influence of the diluting fluid—hard or sea water or water containing organic matter; when such points as these are cleared up and tables of figures obtained to which reference may be made and the coefficient figure modified in accordance with the required conditions, then and then only will it be possible to use such narrow standard tests as those we at present utilise as a definite foundation on which to base the solution of the problems of practical disinfection . . . We believe that it is along these lines that future research should be pursued, and that until these details are forthcoming it is, and will be, impossible to determine what dilutions may be used, safely and economically, to attain effective disinfection under the varying and varied conditions met with in practice." These words appeared in *The Lancet* in 1909 (vol. ii, p. 1615), 52 years ago; they might almost have been written yesterday.

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AN IMPROVED DIAGNOSTIC AND SELECTIVE MEDIUM FOR ISOLATING COAGULASE POSITIVE STAPHYLOCOCCI

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SUMMARY: This paper describes the development and use of a selective and diagnostic medium (ETGPA) for isolating coagulase positive staphylococci from food. It combines an improved form of the selective medium of Zebovitz, Evans & Niven (1955) with the ability to clear egg yolk as a diagnostic character. The new medium is noninhibitory but selective to coagulase positive staphylococci from a variety of sources, and its performance is better than that of four recently published media.

The coagulase positive staphylococci (*Staphylococcus aureus*; Shaw, Stitt & Cowan, 1951) form a well defined group comprising the enterotoxic and potentially pathogenic members of the genus *Staphylococcus*. The isolation of *Staph. aureus* from food is frequently made difficult by the presence of large numbers of other bacteria often belonging to species closely related to this organism. In order to prevent the growth of these it is necessary to use either a selective medium or a combined selective and diagnostic medium, on which colonies of *Staph. aureus* can readily be identified. Many selective and diagnostic media have been described. Media that have been used in this laboratory include those described by Chapman (1946), Maitland & Martyn (1948), Ludlam (1949), Barber & Kuper (1951), Kennedy & Barbara (1952), Zebovitz, Evans & Niven (1955), Klemperer & Haughton (1957), Carter (1960), Innes (1960), Sevel & Plommet (1960) and Finegold & Sweeny (1961). When tested on a variety of foods most of these media were either inhibitory to some strains of *Staph. aureus*, or insufficiently selective or diagnostic to separate this species from the many nonpathogenic staphylococci and micrococci found in foods; of these, strains of *Staph. saprophyticus* (Shaw *et al.* 1951) were a particular nuisance. This species is closely related to *Staph. aureus* but is distinguished by its lack of coagulase activity. Some strains form colonies indistinguishable from *Staph. aureus* on such media as Chapman's 110 medium (Chapman, 1946), Zebovitz *et al.* (1955) medium, Carter's (1960) medium and Innes' (1960) medium. They may also hydrolyze phenolphthalein diphosphate, form acid from mannitol and give the Stone reaction (Stone, 1935). These characters are supposedly diagnostic for coagulase positive staphylococci.

The unreliability of most of the published methods for isolating *Staph. aureus* led to a search for a more specific medium on which this organism could be isolated and enumerated within 24 hr. Hopton (1961) found that *Staph. aureus* cleared 5% egg yolk contained in Difco azide-blood agar base. The appearance of the colonies was thought to be characteristic; they were surrounded by completely clear zones.

These could readily be distinguished from colonies of *Staph. saprophyticus* which had an inner opaque zone surrounded by a clear outer zone. It was found, however, that some strains of *Staph. aureus* formed similar opaque zones after incubation for 24–48 hr (see Carter, 1960; Innes, 1960) and some egg yolk clearing strains of *Staph. saprophyticus* failed to develop inner opaque zones. Various selective media were then tested which suppressed the growth of these confusing organisms and a modified form of the medium of Zebovitz *et al.* (1955) with the addition of egg yolk as a diagnostic agent was developed.

METHODS

Preliminary experiments

Tests on the tellurite, glycine medium of Zebovitz *et al.* (1955) confirmed previous observations by Hoeprich, Croft & West (1960), Sevel & Plommet (1960) and Innes (1960) that it is inhibitory to many strains of *Staph. aureus* on primary isolation. However, it had the advantage of preventing the growth of most egg yolk clearing strains of *Staph. saprophyticus*. The toxicity of the medium to the growth of *Staph. aureus* was reduced by adjusting the tellurite and glycine concentrations to the levels suggested by Sevel & Plommet (1960), i.e. by reducing the concentration of tellurite from 0·2% to 0·0075% and increasing that of the glycine from 1·0% to 1·5% (all as % w/v). Growth was further improved by the separate addition of glycine and tellurite from Seitz filtered solutions after heat sterilization of the basal medium. However, this medium (MTG) was still inhibitory to small inocula of *Staph. aureus* and permitted the growth of some coagulase negative staphylococci, yeasts and an occasional *Bacillus* sp.

The next stage in our investigation was to add various stimulatory compounds to the modified medium in order to improve the growth of *Staph. aureus* without destroying its selectivity. Compounds tested included a wide range of carbohydrates, acids associated with the tricarboxylic acid cycle and miscellaneous compounds such as catalase; of these only sodium pyruvate stimulated growth. At concentrations of 0·25–1·0% (w/v) pyruvate removed all inhibition to the growth of *Staph. aureus*. In a series of experiments with MTG containing 1·0% (w/v) of pyruvate the selectivity of the medium was improved by increasing the tellurite concentration to 0·01% (w/v) and reducing the glycine to 1·2%. Experiments with different brands of potassium tellurite showed wide variations in the toxicity of commercially available tellurites, but different batches of B.D.H. potassium tellurite gave consistent results. The addition of 5% (v/v) of Oxoid concentrated egg yolk emulsion resulted in an opaque medium on which the characteristic clearing activity of *Staph. aureus* could be demonstrated, but initial experiments with this medium were disappointing as the degree of clearing was poor compared with that on a nonselective medium, although colony size was similar. Reduction of the egg yolk concentration had no effect on the size of the clear zones. However, it was later found that the presence of a fermentable carbohydrate reduced the size of these zones, and by omitting mannitol from the MTG medium better clearing was obtained. Phosphates, found by Ludlam (1949) to reduce the toxicity of tellurite, were included in the modifications of this medium described by Zebovitz *et al.* (1955), Sevel & Plommet (1960) and Innes (1960). In the presence of pyruvate, however, phosphates had no stimulatory effect and were not tested further. The final agar medium containing egg yolk, tellurite, glycine and pyruvate was labelled ETGPA, in accordance with the initial letters of its main diagnostic, selective and stimulatory agents.

Final medium

Composition and preparation. The basal medium contained (% w/v): Bacto tryptone, 1·0; Lab-Lemco, 0·5; Bacto yeast extract, 0·1; lithium chloride, 0·5; and agar, 2·0. The constituents were dissolved in distilled water and the pH adjusted to 7·2 before dispensing unfiltered in 90 ml amounts and autoclaving at 120° for 15 min. To the molten basal medium at 45–50° were successively added warmed (45–50°) Seitz filtered solutions of the following: 20% (w/v) glycine (Hopkin & Williams) to a final concentration of 1·2% (w/v); 1·0% (w/v) potassium tellurite (B.D.H.) to 0·01% (w/v); 20% (w/v) sodium pyruvate (L. Light) to 1·0% (w/v); and concentrated egg yolk emulsion (Oxoid) to 5·0% (v/v).

Storage. The basal medium could be stored at room temperature in screw capped bottles for at least two months without loss of selectivity or increased toxicity to *Staph. aureus*. Stock solutions of 20% glycine and 1% tellurite could be stored for a similar period at room temperature. The pyruvate was best stored at 5° and replaced every month. The combination of egg yolk, glycine, tellurite and pyruvate into a mixture or into several mixtures resulted in loss of selectivity. Poured plates of the medium could not be stored satisfactorily, so they were freshly prepared and used within 24 hr of pouring.

Use. Diluted food macerate (0·1 ml) was spread over the surface of well dried plates with a glass spatula. By drying plates at 30° for 18 hr or at 40° for 4 hr up to 1 ml of macerate could be plated. Incubation was at 37° and the results were read after 24–26 hr. The plates were re-incubated for a further 24 hr if no colonies of *Staph. aureus* were apparent. Poured plates could not be used as submerged colonies of some strains of *Staph. aureus* gave atypical egg yolk reactions.

RESULTS

The growth of pure cultures of Staph. aureus *on ETGPA*

The growth of 18 strains of *Staph. aureus* on ETGPA was compared with that on yeast glucose agar (YGA), a medium routinely used in our laboratories. Broth cultures 24 hr old were serially diluted in 0·1% peptone water, 0·1 ml amounts of decimal dilutions plated in triplicate on ETGPA and YGA, the plates incubated at 37° for 24 hr and the colonies counted.

The results, shown in Table 1, indicate that ETGPA is noninhibitory to the growth of *Staph. aureus* strains from a wide variety of sources. The appearance of *Staph. aureus* on this medium was characteristic (Plate 1). After 24 hr at 37° black, shiny, convex colonies 1·0–1·5 mm in diam. were formed, with narrow white entire margins and surrounded by clear zones extending 2–5 mm into the opaque medium. Strains O113 and O119, both isolated from cases of cow mastitis, showed well defined

Table 1. *A comparison of the growth of* 18 *pure cultures of* Staph. aureus *on* ETGPA* *and* YGA*

Strain no.	Source	Count (10^8/ml of broth) on	
		ETGPA*	YGA*
170	Human hand	12·2	12·5
E_1	Human eye	1·91	1·09
38	Food	9·53	9·6
40	Food	4·5	4·5
BSa_3	Food	1·2	1·15
W/2A	Pig skin	12·6	12·4
W/35Y	Pig skin	11·5	12·2
1/P4	Pig skin	2·6	2·78
O112†	Milk (mastitis)	4·1	4·4
O113†	Milk (mastitis)	2·8	3·0
O119†	Milk (mastitis)	3·9	3·8
1236‡	Cow teat	4·15	3·9
1238‡	Cow teat	1·6	1·47
11117	NCTC§	3·0	4·0
11170	NCTC	10·3	10·1
11159	NCTC	3·87	2·15
11195	NCTC	9·0	8·6
11204	NCTC	8·4	8·9

* ETGPA, egg yolk–tellurite–glycine–pyruvate agar; YGA, yeast–glucose agar. † Received from F. K. Neave (N.I.R.D., Shinfield). ‡ 'Unusual types' of Garvie, Higgs & Neave (1961). § NCTC, National Collection of Type Cultures.

clearing only after incubation for 36 hr at 37°. These strains together with NCTC 11208 were the only late egg yolk clearers encountered out of a total of 90 strains tested.

A comparison of ETGPA with four other media for isolating Staph. aureus *from foods*

ETGPA was compared with the Barber & Kuper (1951) phenolphthalein diphosphate agar (A), the Zebovitz *et al.* (1955) tellurite glycine agar (B), the Sevel & Plommet (1960) modification of the Zebovitz *et al.* (1955) medium containing the Camp factor as a diagnostic character (C) and the Innes (1960) tellurite egg yolk agar (D). Duplicate plates of these media were incubated with diluted macerates of two food products and incubated for the times recommended by their authors. Colonies having the

Table 2. *A comparison of ETGPA with* 4 *other media for isolating coagulase positive staphylococci from foods*

Food product	Mean count (10^3/g of food) of presumed coagulase positive staphylococci, and level of confirmation, on media*				
	A	B	C	D	ETGPA
Meat	28 (6/10)†	$<10^2$	4 (5/8)	10 (8/10)	7 (10/10)
Fish	106 (4/10)	10 (8/10)	8 (6/10)	29 (7/10)	12 (10/10)

* A, Barber & Kuper (1951); B, Zebovitz *et al.* (1955); C, Sevel & Plommet (1960) modification of B; D, Innes (1960); ETGPA, egg yolk–tellurite–glycine–pyruvate agar.

† The figures in parenthesis show the numbers of colonies confirmed as coagulase positive out of the number tested.

appearance of *Staph. aureus* were scored as presumed coagulase positive staphylococci and the identity of the colonies confirmed by the tube coagulase test (Gould, 1960).

The results, presented in Table 2, show that the apparent numbers of presumed coagulase positive staphylococci in the two foods varied considerably according to the medium used. The counts on medium A were relatively very large, particularly in the fish product; but out of a total of 20 presumed coagulase positive colonies picked from this medium, only 10 could be confirmed (50% correct). The counts on medium B were low, as also were those on medium C, and the latter, which was developed by Sevel & Plommet (1960) for isolating potentially pathogenic staphylococci forming α-haemolysin, cannot be recommended for isolating coagulase positive staphylococci from foods because some of the isolates were nonhaemolytic on it. However, the error of this medium appears to be smaller than that of medium A because out of 18 isolates tested 11 were coagulase positive (61% correct). Innes' medium (D) gave counts similar to those on ETGPA with the meat product but a much higher and less accurate count with the fish product. Out of 20 isolates on medium D, 15 were coagulase positive (75% correct), whereas all 20 isolates from medium ETGPA were coagulase positive (100% correct).

Coagulase negative organisms on ETGPA

Strains of *Staph. saprophyticus* occasionally grew as black colonies on ETGPA and cleared the egg yolk. However, it was always possible to distinguish this species from *Staph. aureus* by the more irregular appearance of its colonies which, after incubation at 37° for 24 hr, were surrounded by wide opaque zones extending into the cleared medium. Some strains of *Staph. aureus* developed similar zones, but only after incubation for 48 hr or longer. Other organisms occasionally found growing on ETGPA were coagulase negative staphylococci which did not clear the egg yolk and micrococci which formed minute colonies of various shades of brown and black. Yeasts forming white colonies and some *Bacillus* spp. were also occasionally encountered. The latter sometimes cleared the egg yolk after 48 hr but were distinguished by their dark brown matt colonies.

DISCUSSION

There is no established method for demonstrating *in situ* the production of enterotoxin by staphylococci multiplying in foods, although recently an interesting method using nematodes was described by de Valle & McCoy (1960). Until such a method has been proved reliable the only means of demonstrating enterotoxin is by isolation of the organism in question and testing its ability to form the toxin. It is generally agreed that the coagulase test is the best means of detecting potentially enterotoxic staphylococci in the laboratory (Evans & Niven, 1950). Not all coagulase positive staphylococci (*Staph. aureus*) are enterotoxin producers (Dack, 1937), and there is a report by Thatcher & Simon (1957) that coagulase negative organisms may sometimes form enterotoxins. It is difficult conclusively to identify enterotoxic strains without feeding them to human volunteers (Dack, 1937): such tests as the kitten test (Dolman & Wilson, 1940) and feeding to monkeys have been criticized (Fulton, 1943; Elek, 1959). There is also evidence that nonenterotoxic staphylococci will cause food poisoning symptoms in both cats and monkeys (Casman, 1960). Phage typing is a valuable means of identifying enterotoxic strains of staphylococci (Allison, 1949), but only when the phage type has been isolated from a food incriminated in a food poisoning outbreak (Elek, 1959). The *in vitro* enterotoxin test of Levi, Matheson & Thatcher (1956) awaits confirmation, as does the suggested correlation between ability to cause opacity in egg yolk broth and enterotoxin production (Innes, 1960). If the latter relationship is confirmed it could be said that enterotoxic types are mainly of human origin, as most strains of *Staph. aureus* isolated from animal sources fail to cause opacity when grown in egg yolk broth (Alder, Gillespie & Herdan, 1953; Williams Smith, 1954).

The lack of a simple and conclusive test for enterotoxin production leads one to suggest that with our present knowledge it is safer to regard all coagulase positive staphylococci as potentially able to cause food poisoning and to develop media for isolating all the phage and physiological types of these organisms. This was the aim in the development of the ETGPA medium described in this paper. This medium uses as a diagnostic character the ability of coagulase positive staphylococci to cause clearing on an opaque egg yolk medium (Hopton, 1961). This activity is confined mainly to strains of *Staph. aureus* and a few strains of the closely related *Staph. saprophyticus*, both of which are of parasitic origin. The opacity of egg yolk agar was found to depend largely on the sodium chloride content of the medium: increasing this above 1·0% (w/v) resulted in a semiclear medium such as that described by Colbeck (1956), Carter (1960) and Innes (1960). This clearing was presumably due to solubilization of the egg lecithoprotein, since according to Deuel (1951) these compounds are dissolved by the addition of sodium chloride. Staphylococci showed two activities on opaque egg yolk agar. First, clearing of the egg yolk by proteolysis (Willis, 1960) or lipolysis (Richou, Richou, Quinchon & Pantaléon, 1960), and second, the development of opaque zones within the clear zones. These develop within 24 hr in the case of egg yolk clearing strains of *Staph. saprophyticus* and after longer incubation in the case of many strains of *Staph. aureus*. The production of these opaque zones correlated with ability to cause opacity in egg yolk broth. Both are probably the result of a lipase (Gillespie & Alder, 1952; Willis, 1960), although lecithinase activity was reported by Richou *et al.* (1960).

The stimulation by sodium pyruvate of *Staph. aureus* growing on a tellurite glycine agar does not appear to have been reported previously. Current experiments on this stimulatory effect show that whereas *Staph. aureus* appears to metabolize pyruvate many other organisms, e.g. micrococci, metabolize it slowly or not at all (Baird-Parker, unpublished data).

ETGPA has been successfully used in several laboratories. The main drawbacks are that four solutions have to be added to the medium before use and that plates of the medium cannot be stored. However, these disadvantages are outweighed by the great specificity of the medium, which enables coagulase positive staphylococci to be detected at levels as low as 10^1–10^2/g.

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REFERENCES

ALDER, V. G., GILLESPIE, W. A. & HERDAN, G. (1953). Production of opacity in egg-yolk broth by staphylococci from various sources. *J. Path. Bact.* **66**, 205.

ALLISON, V. D. (1949). Discussion on food poisoning. *Proc. Roy. Soc. Med.* **42**, 214.

BARBER, M. & KUPER, S. W. A. (1951). Identification of *Staphylococcus pyogenes* by the phosphatase reaction. *J. Path. Bact.* **63**, 65.

CARTER, C. H. (1960). Egg yolk agar for isolation of coagulase positive staphylococci. *J. Bact.* **79**, 753.

CASMAN, E. P. (1960). Further serological studies of staphylococcal enterotoxin. *J. Bact.* **79**, 849.

CHAPMAN, G. H. (1946). A single culture medium for selective isolation of plasma-coagulating staphylococci, and for improved testing of chromogenesis, plasma coagulation, mannitol fermentation, and the Stone reaction. *J. Bact.* **51**, 409.

COLBECK, J. C. (1956). Studies in hospital infections. 1. The importance of fomites in the spread of staphylococcal infections, with particular reference to mattress and washing facilities. *Canad. Services med. J.* **12**, 563.

DACK, G. M. (1937). Staphylococci in relation to food poisoning. *Amer. J. Publ. Hlth* **27**, 440.

DEUEL, H. J. (1951). *The Lipids*, Vol. 1, p. 417. New York: Interscience Publishers Inc.

DE VALLE, R. & MCCOY, E. (1960). Worms test for poison from 'Golden Staph'. *Science News Letter* Oct. 1st, 1960.

DOLMAN, C. E. & WILSON, R. J. (1940). The kitten test for staphylococcus enterotoxin. *Canad. publ. Hlth J.* **31**, 68.

ELEK, S. D. (1959). Staphylococcus pyogenes *and its Relation to Disease*. London: E. & S. Livingstone.

EVANS, J. B. & NIVEN, C. F. (Jr.) (1950). Study of food poisoning staphylococci. *J. Bact.* **59**, 545.

FINEGOLD, S. M. & SWEENY, E. E. (1961). New selective and differential medium for coagulase positive staphylococci allowing rapid growth and strain differentiation. *J. Bact.* **81**, 636.

FULTON, F. (1943). Staphylococcal enterotoxin with special reference to the kitten test. *Brit. J. exp. Path.* **24**, 65.

GARVIE, E. I., HIGGS, T. M. & NEAVE, F. K. (1961). Unusual varieties of *Staphylococcus aureus*. *J. Path. Bact.* **81**, 255.

GILLESPIE, W. A. & ALDER, V. G. (1952). Production of opacity in egg-yolk media by coagulase positive staphylococci. *J. Path. Bact.* **64**, 187.

GOULD, J. C. (1960). In Mackie and McCartney's *Handbook of Bacteriology*, 10th ed. London: E. & S. Livingstone.

HOEPRICH, P. D., CROFT, C. F. & WEST, L. M. (1960). Tellurite reduction as an indicator of potentially pathogenic staphylococci. *J. Lab. clin. Med.* **55**, 120.

HOPTON, J. (1961). A selective medium for the isolation and enumeration of coagulase positive staphylococci from foods. *J. appl. Bact.* **24**, 121.

INNES, A. G. (1960). Tellurite–egg agar, a selective and differential medium for the isolation of coagulase positive staphylococci. *J. appl. Bact.* **23**, 108.

KENNEDY, E. R. & BARBARA, J. F. (1952). The inhibitory effect of triphenyl-tetrazolium on some strains of micrococci. *J. Bact.* **63**, 297.

KLEMPERER, R. & HAUGHTON, G. (1957). A medium for the rapid recognition of penicillin-resistant coagulase-positive staphylococci. *J. clin. Path.* **10**, 96.

LEVI, L., MATHESON, B. H. & THATCHER, F. S. (1956). Detection of staphylococcus enterotoxin by infra-red spectrophotometry. *Science* **123**, 64.

LUDLAM, G. B. (1949). A selective medium for the isolation of *Staphylococcus aureus* from heavily contaminated material. *Mon. Bull. Ministr. Hlth Lab. Serv.* **8**, 15.

MAITLAND, H. B. & MARTYN, G. (1948). A selective medium for isolating staphylococci based on the differentially inhibiting effect of increased concentrations of sodium chloride. *J. Path. Bact.* **60**, 553.

RICHOU, R., RICHOU, R., QUINCHON, C. & PANTALÉON, J. (1960). Sur la présence dans les filtrats de culture du staphylocoque, d'enzymes attaquant les lipids (graisse neutre, ovolipids, lécithine), et la caseine. *Rev. Immunol,* **24**, 496.

SEVEL, B. & PLOMMET, M. (1960). Adaptation d'un milieu selectif à l'isolement des staphylocoques de mammite. Application au diagnostic d'étable. *Le Lait* **40**, 2.

SHAW, C., STITT, J. M. & COWAN, S. T. (1951). Staphylococci and their classification. *J. gen. Microbiol.* **5**, 1010.

SMITH, H. WILLIAMS (1954). The pathogenicity and haemolytic properties of staphylococci isolated from chickens. *J. Path. Bact.* **67**, 73.

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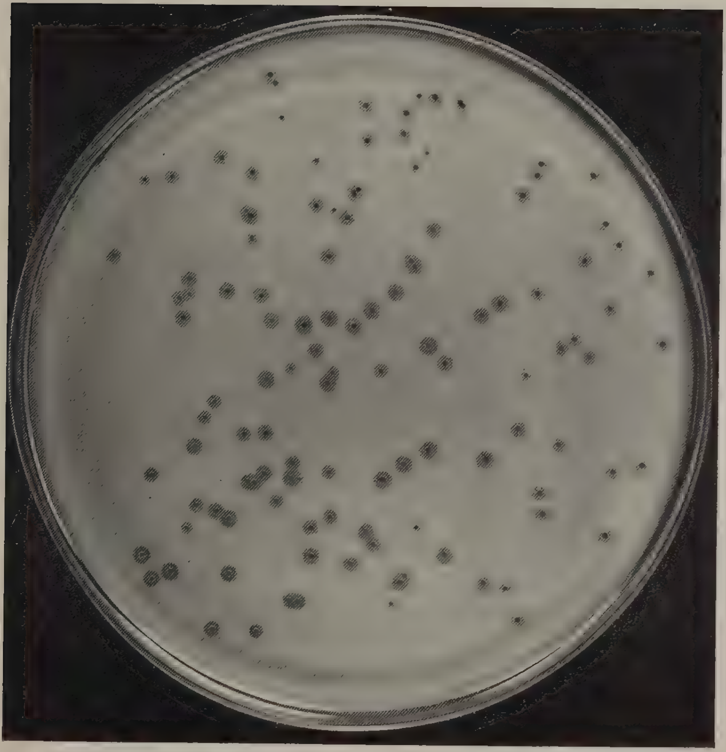

A. C. Baird-Parker — An Improved Diagnostic and Selective Medium for Isolating Coagulase Positive Staphylococci. Plate 1

STONE, R. V. (1935). A cultural method for classifying staphylococci as of the 'food-poisoning' type. *Proc. Soc. exp. Biol., N.Y.*, **33**, 185.

THATCHER, F. S. & SIMON, W. (1957). Some physiological and toxigenic properties of members of the genus *Micrococcus* in relation to taxonomy. *Int. Bull. bact. Nomen. Taxon.* **7**, 21.

WILLIS, A. T. (1960). The lipolytic activity of some clostridia. *J. Path. Bact.* **80**, 379.

ZEBOVITZ, E., EVANS, J. B. & NIVEN, C. F. (Jr.) (1955). Tellurite–glycine agar: a selective plating medium for the quantitative detection of coagulase-positive staphylococci. *J. Bact.* **70**, 686.

EXPLANATION OF PLATE

The appearance of a pure culture of *Staph. aureus* on ETGPA after incubation at 37° for 24 hr $\times\frac{3}{5}$.

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Mossel, D. A. A. (1962). *J. appl. Bact.* **25** (1), 20–29.

AN ECOLOGICAL INVESTIGATION ON THE USEFULNESS OF TWO SPECIFIC MODIFICATIONS OF EIJKMAN'S TEST AS ONE OF THE METHODS USED FOR THE DETECTION OF FAECAL CONTAMINATION OF FOODS

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SUMMARY: An investigation has been carried out on the suitability of two modifications of Eijkman's heat tolerance test which had previously been found useful for the detection of *Escherichia coli* strains of faecal origin in foods. These were: plating on Chapman's lactose tergitol-triphenyltetrazolium agar incubated at 44°, and enrichment in brilliant green-bile fluid incubated at 44°, followed by subculture in the same medium and in tryptone water to test indole formation. Two materials having quite different histories of faecal contamination were examined. These were 'coliform positive' ice cream samples and bulk raw human milk. The reliability of both methods was found to be very satisfactory.

In spite of this, such Eijkman tests appeared to have only limited usefulness because *E. coli* was found to be readily outgrown by other bacteria under extraenteral conditions; *E. coli* was therefore absent relatively often even from 1 ml samples of human milk which contained quite high numbers (i.e. $>10^3$/ml) of other organisms of faecal origin. It is recommended, therefore, that too much reliance should not be placed on the outcome of Eijkman tests, especially when they give a negative result. The hygienic evaluation of foods should rather be based on the results of the simultaneous enumeration of three groups of organisms, namely *E. coli*, faecal types of *Klebsiella* (i.e. those which are urease positive) and Lancefield group D streptococci.

A striking result of this investigation was the occurrence in 69% of the human milk samples of 44° positive *Achr. anitratus* (B5W) in numbers from $10–5{\cdot}6\times10^3$/ml. These bacteria were almost invariably found in samples which were *E. coli* negative; klebsiellae, many of which were *Kb. oxytoca*, were also found in such samples. The properties of these organisms and their ecological significance are discussed.

For the estimation of the hygienic condition of foods not subjected to a final heat treatment the detection of *Escherichia coli* strains of faecal origin is generally considered essential, while this test is helpful in the examination of heat treated foods (Mossel, 1956). Such faecal strains of *E. coli* (*E. coli* type I, Report, 1956) are mostly sought with the help of Eijkman's (1904) test. The poor results recently obtained by Raj & Liston (1961) and Tennant *et al.* (1961) with Hajna & Perry's modification (1943) of that test have led us to reinvestigate this differential reaction, because it had invariably given sufficiently reliable results in the past (Bekker & Mossel, 1955; Buttiaux & Mossel, 1957; Mossel, 1961). It should be made clear right from the beginning, however, which modifications of Eijkman's test we refer to because, as has been demonstrated by Batty-Smith (1942), experimental factors such as choice of sugar, presence of a buffer and various inhibitors and choice as well as exact control of the temperature of incubation, greatly influence the outcome of the test. The modifications used by us are as follows.

First, the one suggested by Mackenzie, Taylor & Gilbert (1948) in which parallel subcultures are made in brilliant green-bile broth and tryptone water at 44±0·1°. The reliability of this procedure has been established as satisfactory by Thomas *et al.* (1955) in Great Britain, in France by Buttiaux, Samaille & Pierens (1956) as well as by Panteléon, Galanis & Guibert (1959), and also in various Mediterranean countries (Papavassiliou, 1958; Graziadei-Celoria, 1958).

Second, the test elaborated by Buttiaux, Muchemblé & Leurs (1953) in which the food sample under investigation is subcultured on plates of Chapman's (1951) lactose-tergitol-triphenyltetrazolium chloride-bromothymol blue (LTTB) agar incubated at 44°. This procedure has been found a very effective one by Bekker & Mossel (1955) and by Seigneurin & Magnin (1957), although it clearly has the disadvantage of permitting the examination of only relatively small volumes of material.

The materials chosen for examination were such that they were probably different from an ecological standpoint. The first was ice cream which had already been shown (Mossel, Hensel & de Bruin, 1958), at least in The Netherlands, to be predominantly infected by inadequately cleaned apparatus (cf. Abrahamson & Clinton, 1960). Because it is well known that *E. coli* is the least fast growing under extraenteral conditions of all the various lactose positive Enterobacteriaceae (Thomas *et al.* 1960) it is plausible that ice cream samples showing a positive 'coliform' test would contain predominantly *Enterobacter*, *Citrobacter* and *Klebsiella* spp. with *E. coli* occurring only rarely. The other material investigated was bulk raw human milk, known to be generally heavily contaminated with various bacteria of strictly human origin (Mossel & Weijers, 1957), and therefore probably richer in faecal *E. coli* than foods, only remotely infected with human material.

MATERIALS AND METHODS

Ice cream

The ice cream samples examined were obtained from the Municipal Food Inspection Laboratory at Utrecht, The Netherlands. They had been stored at +2° since sampling and were 'coliform' positive according to The Netherlands Ice Cream Regulation, i.e. 0·1 ml quantities in brilliant green-bile broth formed gas within 48 hr at 37°.

Human milk

The bulk raw human milk samples had been made available by the Regional Human Milk Bank at the Pasteur Institute, Lille, France. Each sample contained milk from 10–20 different donors, bulking being done only after the pH had been shown to be normal. The samples were taken just before being pasteurized and were examined immediately or after storage for 24 hr at a temperature not exceeding +2°.

General bacteriological examination of samples

All samples were examined for the following bacteriological characters: the total aerobic plate count in TDYM agar (Mossel & Krugers Dagneaux, 1959); the numbers of viable Lancefield group D streptococci in Packer's (1943) crystal violet-sodium azide-blood agar (Mossel, van Diepen & de Bruin, 1957); the numbers of viable *Staphylococcus aureus* on Chapman's (1945) mannitol-salt-phenol red agar (Mossel & Vendrig, 1956), confirmation of mannitol positive colonies being carried out by stabbing into tubes of mannitol agar (Evans, 1948; Mossel, 1962); and the approximate numbers of nonfermentative Gram-negative rods (*Pseudomonas*, *Achromobacter* and similar species) by plating on Olson's (1961) crystal violet agar, confirmation of organisms presumed not to be Enterobacteriaceae being carried out by stabbing into tubes of glucose agar (Mossel & Martin, 1961).

Examination for different groups of Enterobacteriaceae

Total Enterobacteriaceae. Viable bacteria of this group were enumerated by plating 1 ml quantities of suitable dilutions (i.e. those yielding between 20 and 200 colonies) in violet red-bile-mannitol agar (Mossel, 1957). A representative number of colonies obtained after incubation for about 20 hr at 37° was examined in the so-called GNiLUMoCISO test of Mossel (1961) for detection of the following characters: metabolism of glucose (G) and lactose (L) in deep culture so as to recognize oxidative as well as fermentative attack on these sugars (Hugh & Leifson, 1953; Mossel & Martin, 1961); reduction of nitrate (Ni); hydrolysis of urea (U); motility (Mo); assimilation of citrate (C); formation of indole (I); desulphydration of thiosulphate (S); and the oxidase test (O) to preclude mistaking *Aeromonas* for Enterobacteriaceae (Buttiaux & Gagnon, 1959; Eddy, 1960). Organisms isolated and characterized in this way were identified according to the more recent suggestions of Hugh (1959), Ewing & Edwards (1960), Cowan *et al.* (1960) and Hormaeche & Edwards (1960).

Modified Eijkman test for E. coli. Portions of 1 ml and 0·1 ml of ice cream and human milk were enriched in 10 ml of brilliant green-bile broth by incubating for 24–48 hr (depending on the rapidity of gas formation) in an electronically controlled water bath at 44±0·1°. Tubes showing gas production were subcultured into brilliant green-bile broth and into tryptone water, and incubated at 44°. If gas was formed in the former and indole appeared present in the latter after 48 hr, i.e. when the Mackenzie, Taylor & Gilbert (1948) test was positive, the dominant strain was isolated from the brilliant green-bile tube by plating on Chapman's LTTB agar and incubating in an incubator regulated at 44±0·3° by means of forced air circulation. Isolates obtained from such plates were tested for identity with *E. coli* by carrying out the IMViC tests plus a test for the formation of indole at 44°. Fouad & Richard's (1953) medium was used for the methyl red and Voges-Proskauer tests; citrate assimilation was studied on slants of Simmons' (1926) agar at 30°.

Direct counts of faecal E. coli *on plates.* Portions of 0·1 ml of the samples were transferred to the centre of plates of Chapman's LTTB agar contained in Petri dishes of 15 cm diameter and evenly spread over these plates by means of sterile bent glass rods. Inoculated plates were stored for about 20 hr in the ventilated 44° incubator, then TTC negative but lactose positive colonies were counted and recorded as presumptive faecal *E. coli*. Colonies having this appearance are mostly formed only by these bacteria, although exceptions are slightly more common than with the modified Eijkman test according to Mackenzie *et al.* (1948) and Buttiaux *et al.* (1953).

RESULTS AND DISCUSSION

Results of ecological significance

Comparison of human milk and ice cream. The results obtained are presented in Tables 1 and 2. In no case were 0·1 ml samples (that is 1 ml of 10^{-1} dilutions) of ice cream positive even in the first brilliant green-bile test, whereas only 4 out of the 50 samples examined appeared to contain faecal *E. coli* in 1 ml volumes; yet, according to total and specific counts, the ice cream samples were generally severely contaminated. This clearly shows that the Enterobacteriaceae found in rejected samples of Dutch ice cream mostly arise from environments which have probably been infected initially by the human hand or by faecally contaminated raw materials, but in which the *E. coli* strains were subsequently outgrown by other species. In this material these were mainly *Klebsiella* organisms (47% of isolates), *Citrobacter freundii* (23%), *Hafnia alvei* (15%) and *Enterobacter cloaca* (15%).

Table 1. *The incidence of different groups of bacteria in ice cream and human milk*

Material and no. of samples examined	$\log_{10}$ (average counts/ml) of			
	Total organ-isms	Faecal strepto-cocci	*Staph. aureus*	Entero-bacteri-aceae
Ice cream 50	5·33	2·04	1·06	3·40
Human milk 50	5·84	4·76	2·24	5·14

The situation was entirely different with the human milk samples. First, although once again the predominant Enterobacteriaceae were not *E. coli*, *Klebsiella* organisms were much more frequent than in ice cream, occurring in numbers over 10^3/ml in 85% of samples and comprising 59% of isolates other than *E. coli*; *Enterobacter* spp. were also numerous among the isolates (40% of samples and 26% of isolates). Next, it was possible to detect faecal *E. coli* in 41 out of 50 samples, 34 of which contained the organism in 0·1 ml volumes, the smallest quantities tested. The higher average count of total Enterobacteriaceae in the human milk samples compared with the samples of ice cream, the $\log_{10}$ (average counts/ml) being 5·14 and 3·40 respectively, does not account for this difference in frequency of isolation of *E. coli*. In fact the $\log_{10}$ (average count of the presumptive *E. coli*/ml) on LTTB agar at 44° was 2·62 for the human milk samples. If this is corrected to the same denominator as the total Enterobacteriaceae count in the ice cream samples, a figure of 0·88 results. This means that, if the ice cream samples had been as severely infected with *E. coli* as the human milks, most of the 1 ml volumes tested would have been positive in the Eijkman test, which was not the case.

Therefore, although the faecal *E. coli* had been outgrown by other Enterobacteriaceae in the human milk during the storage before it was taken to the Human Milk Bank (Mossel & Weijers, 1957), these organisms were still present in considerable numbers. In the ice cream, however, *E. coli* had almost entirely disappeared as a result of being exposed to extraenteral conditions on two occasions, namely in the focus of infection from which the ice cream was contaminated and in the ice cream itself.

Table 2. *The incidence of different groups of bacteria in ice cream and human milk**

Material and no. of samples examined	Incidence of									
	Enterobacteriaceae*, (% of isolates)					Eijkman +ve (% of samples) in		Other bacteria of ecological significance (% of samples)		
	Alkalescens	*Citr. freundii*	*Klebsiella*	*Enterobacter*	*Hafnia*	1 ml	0·1 ml	*Kb. oxytoca*	*Achr. anitratus*	*Aeromonas*
Ice cream 52	0	23	47	15	15	8	0	—	—	—
Human milk 50	1	3	59	26	11	82	68	51	69	2

* The methods used have been described by Mossel (1961).

Occurrence of Achromobacter anitratus. It is of interest to note that in the course of the examination of human milk samples on LTTB agar lactose negative but strongly TTC reducing colonies were observed quite frequently, i.e. in 69% of the samples. With the help of the GNiLUMoCISO test these were identified as *Achromobacter anitratus*, an organism whose ecology is not yet very well known but which is mostly (Ferguson & Roberts, 1950; Brisou & Prévot, 1954; Billing, 1955; Wilssens & Buttiaux, 1958; Ellis, 1961; Ashley & Kwantes, 1961) isolated from human and animal sources with quite often a history of pathology, although Papavassiliou (1955) and Wilssens & De Vleeschauwer (1960) isolated it from pasteurized cow's milk. It was quite striking that this organism was also invariably found in those samples of human milk which did not contain any (i.e. <1/ml) *E. coli*. A summary of the properties of the strains isolated is given in Table 3.

Table 3. *Some of the properties of* 35 *strains of* Achr. anitratus *isolated from bulk raw human milk**

Test	Percentage of strains giving positive result
Motility or creeping movement†	0
Oxidation of glucose‡	100
Oxidation of lactose‡	0
Oxidation of 10% lactose§	100
Reduction of nitrate to nitrite	0
Assimilation of citrate	100
Formation of indole	0
Formation of H_2S in cysteine broth	0
Inhibition of growth by KCN	100
Oxidase test positive	0
Hydrolysis of urea	26
Hydrolysis of gelatin	74
Growth on 5% soap agar	0

* The methods used have been described by Mossel (1961).

† cf. Lautrop (1961).

‡ Tests done in 8 mm tubes in agar containing 1% of sugar.

§ Tests done on slants of 10% lactose agar.

A few attempts were made to isolate *Achr. anitratus* from stools by plating 0·05 ml quantities of a 10^{-5} dilution on LTTB agar and incubating at 44°. In this way three 44° positive strains of *Achr. anitratus* were isolated from the 36 stools examined. Most TTC positive lactose negative isolates obtained in this way appeared to be lactose negative *E. coli* (‘*Paracolobactrum coliforme*’) strains. Hence the rather prevalent contamination of bulk raw human milk with *Achr. anitratus* may not primarily stem from stools but rather arise from the same focus from which the numerous *Staph. aureus* originate (cf. Table 1).

Klebsiella *strains*. As is shown by Table 2, *Klebsiella* organisms, as defined by Cowan *et al.* (1960), were very frequently isolated from the human milk samples. They were all urease positive, at least when tested on Christensen's (1946) medium, which points to their faecal origin (Buttiaux, Samaille & Pierens, 1956).

A further striking result was the quite frequent occurrence in the human milk samples of the organism *Klebsiella oxytoca* (Hugh, 1959). This organism has rather often been isolated from the human environment, although its ecology is not entirely clear. The frequent coincident occurrence in human milk of *Kb. oxytoca* with *Achr. anitratus*, *Streptococcus faecalis* and rather often *E. coli* is, in our opinion. not without considerable importance from the point of view of ecology.

It should be noted, however, that generally ubiquitous organisms have also been isolated from bulk raw human milk. Among these were *Pseudomonas* spp., found in most of the samples in numbers of the order of 10^6/ml and *Achromobacter* spp., in virtually all samples and in the same numbers. *Aeromonas* strains, however, have been isolated from one sample only (2% of the total material examined).

Reliability of the test of Mackenzie, Taylor & Gilbert (1948)

One of the primary objectives of this study was to test the applicability of Mackenzie, Taylor & Gilbert's (1948) modification of Eijkman's test. In our hands this test proved very useful because of its simplicity, rapidity and, above all, reliability. In fact, in 43 of 45 (i.e. slightly more than 95%) instances, when a milk or ice cream sample showed gas formation in brilliant green-bile broth at 44° and when positive subcultures at 44° in brilliant green-bile and tryptone water were obtained, we have been able to isolate *E. coli* from the enrichment fluids showing a positive Eijkman test. Clearly the brilliant green-bile cultures at 44° often contained in addition other organisms capable of growing at that temperature, so that not all the yellow colonies appearing on the LTTB agar plates made from these cultures were *E. coli*.

Our results therefore confirm the earlier ones obtained by the same technique. This statement, however, has more significance than is at first apparent, because in most previous investigations water or pasteurized milk was tested, whereas the present tests were done on products which were very heavily contaminated (see Tables 1 and 2); in fact, the test of Mackenzie *et al.* quite frequently enabled us to enrich low numbers (*c.* 10/ml) of *E. coli* from a total microflora of well over 10^6/ml.

Reliability of direct plating on LTTB agar

As has been shown by Buttiaux *et al.* (1953) and ourselves (Bekker & Mossel, 1955) the direct plating of foods on LTTB agar at 44° may sometimes yield colonies the same as or quite similar to those of *E. coli* but which are formed by other bacteria. If this procedure is used, therefore, a representative number of colonies must be confirmed: for this confirmation the modification of the Eijkman test as described by Mackenzie *et al.* (1948) is obviously the method of choice.

The advantage of the plating technique is, of course, that in addition to *E. coli* other organisms, especially *Achr. anitratus*, are detected and enumerated in the same operation.

Conclusions

The high reliability of the tested modifications of the Eijkman test, even when applied to severely contaminated foods, does not detract from the fact that *E. coli* appeared to be—in this investigation as in very many previous ones (cf. the review of literature given by Mossel, van Diepen & de Bruin, 1957)—a rather poor indicator of the faecal contamination of foods. In spite of the fact that Lancefield group D streptococci are generally about ten times less numerous than *E. coli* in faeces, they are several times more frequent in contaminated foods because of their much better resistance to extraenteral conditions than *E. coli*. This investigation, however, shows once again that the choice is not merely one between Lancefield-D streptococci and Enterobacteriaceae. It is well known that many pathogenic enteric bacteria are much more resistant to extraenteral conditions than *E. coli*. The *E. coli* is not, therefore, a suitable sole indicator organism for the pathogenic Enterobacteriaceae and still less for the still more robust enteric viruses. The results reported here show that urease positive *Klebsiella* spp. may be more suitable for this purpose because they are very probably of faecal origin and are more resistant than *E. coli*.

It seems that all the evidence available at present points to the necessity of a modified approach to the use of indicator organisms for safeguarding food supplies. The Eijkman test remains a very suitable and reliable test as before; but, if the outcome is negative, it does not necessarily show that the food examined is safe. Attempts should then be made to demonstrate the absence in significant numbers of other organisms of faecal origin such as klebsiellae and group D streptococci. This conclusion, therefore, gives further support to the approach first advocated some four years ago by Buttiaux (1959).

The potential role of *Achr. anitratus* as a further indicator organism in food hygiene has become apparent from this investigation. However, much more ecological research is needed before the criticism which will no doubt be levelled at the suggestion of its use as an indicator of undesired pollution of food can be answered.

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REFERENCES

ABRAHAMSON, A. E. & CLINTON, A. F. (1960). The control of bacterial populations in foods. *Assoc. Food & Drug. Off. U.S. Quart. Bull.* **24**, 31.

ASHLEY, D. J. B. & KWANTES, W. (1961). Four cases of human infection with *Achromobacter anitratus*. *J. clin. Path.* **14**, 670.

BATTY-SMITH, C. G. (1942). The Eijkman test. *J. Hyg., Camb.*, **42**, 55.

BEKKER, J. H. & MOSSEL, D. A. A. (1955). Evaluation of a medium containing tergitol-7 and triphenyltetrazoliumchloride for the differentiation of coliform bacteria from drinking water and foods. *Leeuwenhoek ned. Tijdschr.* **21**, 252.

BILLING, E. (1955). Studies on a soap tolerant organism: a new variety of *Bacterium anitratum*. *J. gen. Microbiol.* **13**, 252.

BRISOU, J. & PRÉVOT, A. R. (1954). Études de systématique bactérienne X. Révision des espèces réunies dans le genre *Achromobacter*. *Ann. Inst. Pasteur* **86**, 722.

BUTTIAUX, R., MUCHEMBLÉ, G. & LEURS, TH. (1953). La colimétrie de l'eau sur membranes filtrantes. *Ann. Inst. Pasteur* **84**, 1010.

BUTTIAUX, R., SAMAILLE, J. & PIERENS, Y. (1956). L'identification des Eschericheae des eaux. Test d'Eijkman et production d'indole à 44°. *Ann. Inst. Pasteur, Lille*, **8**, 137.

BUTTIAUX, R. & MOSSEL, D. A. A. (1957). L'analyse bactériologique des produits alimentaires périssables et conservés. *Ann. Inst. Pasteur, Lille*, **9**, 138.

BUTTIAUX, R. (1959). The value of the association Escherichieae-group D streptococci in the diagnosis of contamination in foods. *J. appl. Bact.* **22**, 153.

BUTTIAUX, R. & GAGNON, P. (1959). Au sujet de la classification des *Pseudomonas* et des *Achromobacter*. *Ann. Inst. Pasteur, Lille*, **10**, 121.

BUTTIAUX, R. & MOSSEL, D. A. A. (1961). The significance of various organisms of faecal origin in foods and drinking water. *J. appl. Bact.* **24**, 353.

CHAPMAN, G. H. (1945). The significance of sodium chloride in studies of staphylococci. *J. Bact.* **50**, 201.

CHAPMAN, G. H. (1951). A culture medium for detecting and confirming *Escherichia coli* in ten hours. *Amer. J. Publ. Hlth* **41**, 1381.

CHRISTENSEN, W. B. (1946). Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. *J. Bact.* **52**, 461.

CLEGG, L. F. L. & SHERWOOD, H. P. (1939). Incubation at 44°C as a test for faecal coli. *J. Hyg., Camb.*, **39**, 361.

COWAN, S. T., STEEL, K. J., SHAW, C. & DUGUID, J. P. (1960). A classification of the *Klebsiella* group. *J. gen. Microbiol.* **23**, 601.

EDDY, B. P. (1960). Cephalotrichous fermentative Gram-negative bacteria: the genus *Aeromonas*. *J. appl. Bact.* **23**, 216.

EIJKMAN, C. (1904). Die Gärungsprobe bei 46° als Hilfsmittel bei der Trinkwasseruntersuchung. *Zbl. Bakt.* (Abt. I), **37**, 742.

ELLIS, E. M. (1961). The recovery of *Bacterium anitratum* (B5 w) from animals. *Amer. J. vet. Res.* **22**, 610.

EVANS, J. B. (1948). Studies on staphylococci with special reference to the coagulase-positive types. *J. Bact.* **55**, 793.

EWING, W. H. & EDWARDS, P. R. (1960). The principal divisions and groups of Enterobacteriaceae and their differentiation. *Int. Bull. Bact. Nom. Taxon.* **10**, 1.

FERGUSON, W. W. & ROBERTS, L. F. (1950). A bacteriological and serological study of organism B5W (*Bacterium anitratum*). *J. Bact.* **59**, 171.

FOUAD, M. T. A. & RICHARDS, T. (1953). The use of glucose inorganic salts media in the classification of the coli-aerogenes bacteria. I. The methyl red and Voges-Proskauer reactions. *Proc. Soc. appl. Bact.* **16**, 35.

GRAZIADEI-CELORIA, M. L. (1958). Valore del test di Eijkman modificato da Mackenzie, Taylor e Gilbert abbinato con la ricerca dell' indolo a 44°C per la rapida identificazione di *E. Coli* nelle acque di alimentazione. *G. Batt. Immun.* **51**, 129.

HAJNA, A. A. & PERRY, C. A. (1943). Comparative study of presumptive and confirmative media for bacteria of the coliform group and for fecal streptococci. *Amer. J. Publ. Hlth* **33**, 550.

HORMAECHE, E. & EDWARDS, P. R. (1960). A proposed genus *Enterobacter*. *Int. Bull. Bact. Nomen. Taxon.* **10**, 71.

HUGH, R. & LEIFSON, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria. *J. Bact.* **66**, 24.

HUGH, R. (1959). Oxytoca group organisms isolated from the oropharyngeal region. *Canad. J. Microbiol.* **5**, 251.

LAUTROP, H. (1961). *Bacterium anitratum* transferred to the genus *Cytophage*. *Int. Bull. Bact. Nomen. Taxon.* **11**, 107.

MACKENZIE, E. F. W., TAYLOR, E. W. & GILBERT, W. E. (1948). Recent experiences in the rapid identification of *Bacterium coli* type I. *J. gen. Microbiol.* **2**, 197.

MOSSEL, D. A. A. & VENDRIG, C. M. A. (1956). The enumeration of *Staphylococcus aureus* in foods. *Leeuwenhoek ned. Tijdschr.* **22**, 205.

MOSSEL, D. A. A. (1956). Aufgaben und Durchführung der modernen hygienisch bakteriologischen Lebensmittelüberwachung. *Wien. tierärztl. Mschr.* **43**, 321.

MOSSEL, D. A. A. & WEIJERS, H. A. (1957). Uitkomsten verkregen bij bacteriologisch onderzoek van vrouwenmelk van diverse herkomst en de betekenis daarvan voor de pediatrische praktijk. *Maandschr. Kindergeneesk.* **25**, 37.

MOSSEL, D. A. A. (1957). The presumptive enumeration of lactose negative as well as lactose positive Enterobacteriaceae in foods. *Appl. Microbiol.* **5**, 379.

MOSSEL, D. A. A., VAN DIEPEN, H. M. J. & DE BRUIN, A. S. (1957). The enumeration of faecal streptococci in foods, using Packer's crystal violet sodium azide blood agar. *J. appl. Bact* **20**, 265.

MOSSEL, D. A. A., HENSEL, H. & DE BRUIN, A. S. (1958). The reliability of the "coliform test" as a criterion for the bacteriological condition of ice-cream. Unpublished Report no. 819. Central Institute for Nutrition and Food Research T.N.O., Utrecht, The Netherlands.

MOSSEL, D. A. A. & KRUGERS DAGNEAUX, E. L. (1959). Bacteriological requirements for and bacteriological analysis of precooked ("instant") cereals and similar foods. *Leeuwenhoek ned. Tijdschr.* **25**, 230.

MOSSEL, D. A. A. (1961). Eine Schnellidentifizierung von Gram-negativen, Stäbchenförmigen Bakterien isoliert bei der hygienisch-bakteriologischen Lebensmitteluntersuchung. *Arch. Lebensm. Hyg.* **12**, 180.

MOSSEL, D. A. A. & MARTIN, G. (1961). Milieu simplifié permettant l'étude des divers modes d'action des bactéries sur les hydrates de carbone. *Ann. Inst. Pasteur, Lille*, **12**, (in press.)

MOSSEL, D. A. A. (1962). An attempt of classification of the catalase positive cocci (staphylococci and micrococci). *Bacteriol. Proc.* **62**, 921.

OLSON, H. C. (1961). Selective media for detecting spoilage organisms in dairy products. *J. Dairy Sci.* **44**, 970.

PACKER, R. A. (1943). The use of sodium azide (NaN_3) and crystal violet in a selective medium for streptococci and *Erysipelothrix rhusiopathiae*. *J. Bact.* **46**, 343.

PANTALÉON, J., GALANIS, N. & GUIBERT, M. (1959). Recherche et dénombrement des *Escherichia coli* dans les produits carnés. Emploi de la gélose au désoxycholate de sodium à température d'incubation de 44°. *Bull. Acad. vét. Fr.* **32**, 467.

PAPAVASSILIOU, J. (1955). *B. anitratum* (B5W) eis pasterioména galakta en` Agglia. *Iatrike* **E 68**, 523.

PAPAVASSILIOU, J. (1958). The rapid identification of *Escherichia coli* I by the production at 44° of both indole and gas from lactose. *J. appl. Bact.* **21**, 104.

RAJ, H. & LISTON, J. (1961). Detection and enumeration of fecal indicator organisms in frozen sea foods. I. *Escherichia coli*. *Appl. Microbiol.* **9**, 171.

REPORT (1956). The nomenclature of coli-aerogenes bacteria. *J. appl. Bact.* **19**, 108.

SCHUBERT, R. (1958). Studien zur Differenzierung coliformer Bakterien in der Wasserdiagnostik. *Z. Hyg. InfektKr.* **144**, 485.

SEIGNEURIN, R. & MAGNIN, R. (1957). Utilisation du milieu Tergitol 7 TTC pour la colimétrie des laits crus et pasteurisés. *Ann. Inst. Pasteur* **93**, 537.

SIMMONS, J. S. (1926). A culture medium for differentiating organisms of the typhoid-colon aerogenes groups and for isolation of certain fungi. *J. infect. Dis.* **39**, 209.

TENNANT, A. D. & REID, J. E. (1961). Coliform bacteria in sea water and shellfish. I. Lactose fermentation at 35·5° and 44°C. *Canad. J. Microbiol.* **7**, 725.

TENNANT, A. D., REID, J. E., ROCKWELL, L. J. & BYNOE, E. T. (1961). Coliform bacteria in sea water and shellfish. II. The E.C. confirmation test for *Escherichia coli*. *Canad. J. Microbiol.* **7**, 733.

THOMAS, S. B., CLEGG, L. F. L., CUTHBERT, W. A., OXLEY, C. D., SCARLETT, C. A., WESTWATER, C. H., JONES, G. E., MCCLEMONT, J., MORRIS, C. S. & NICHOLS, A. A. (1955). Studies of the determination of the *Bacterium coli* type I content of farm water supplies. *J. appl. Bact.* **18**, 9.

THOMAS, S. B., DRUCE, R. G. & ELSON, K. (1960). An ecological study of the coli-aerogenes bacteria of surface soil. *J. appl. Bact.* **23**, 169.

WILSSENS, A. & BUTTIAUX, R. (1958). Les bactéries de la flore fécale de la vache saine. *Ann. Inst. Pasteur* **94**, 332.

WILSSENS, A. & DE VLEESCHAUWER, A. (1960). Some aspects of the microflora of pasteurized milk after holding under refrigeration. *J. appl. Bact.* **23**, iii.

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Lev, M. (1962). *J. appl. Bact.* 25 (1), 30–34.

AN AUTOCLAVABLE PLASTIC UNIT FOR REARING ANIMALS UNDER GERM FREE CONDITIONS

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SUMMARY. A plastic unit for rearing germ free animals is described. This is sterilized by steam under pressure and has been successfully used to rear germ free chicks.

Although germ free animals are now recognized tools in biological research, their use is restricted by the expense of the apparatus involved. For the apparatus previously described, steam under pressure is the sterilizing medium of choice and this consideration has influenced the design. The two main types of apparatus in use are those which are autoclaves and those which are autoclavable. Reyniers, Trexler & Ervin (1946) and Reyniers (1959) described an apparatus which is essentially a stainless steel autoclave modified to take air sterilized by filtration through glass wool. This apparatus has attached to it a small subsidiary autoclave for entry and exit of materials. Most of the experimental work reported has been done with this apparatus modified when necessary for special purposes.

Gustafsson (1948, 1959) has used an elegantly designed light gauge stainless steel apparatus consisting of a box fitted with a plate glass top, entries into which are made via a germicidal trap. Air is sterilized by incineration at 300°.

A plastic unit described by Trexler & Reynolds (1957) has the disadvantage of not withstanding heat sterilization but requiring the use of chemical disinfectants. Gustafsson (1959) has stressed the advantages to be gained by using an autoclavable plastic for the construction of germ free rearing units.

It was felt that the design of a cheap reliable autoclavable plastic unit would be an asset to germ free animal research. Such a unit is described and has been used for the rearing of germ free chicks.

Materials and Methods

Construction of the unit. The unit consists essentially of a nylon tube 8 ft long, 78 in. in circumference and 0·002 in. thick, made from two pieces of 20 in. wide lay-flat Portex (Portland Plastics Ltd., Hythe, Kent) nylon tube cemented together with nylon adhesive. The joint is covered with tape for extra protection. The tube is drawn over an angle-alloy frame 40 in. in length, 21 in. wide at the base, 11½ in. high at the front and 19 in. high at the back (Fig. 1, a and b). One end of the tube is constricted around three stainless steel rings of 8 in. diameter which fit into a germicidal trap consisting of a stainless steel trough 10 in. × 10 in. × 20 in. fitted with a lid to cover the part not occupied by the nylon tube (Fig. 1b, Plate 1). The free end of the nylon tube is used for loading the apparatus with cages, water, etc. and is then sealed by twisting and folding. The fold is tightly wrapped and tied with rubber tubing (Plate 2).

The gloves are of rolled neoprene (28 in. × 6 in. and 0·015–0·022 in. thick, Veedip Ltd., Slough) and are supported on stainless steel rings of 6 in. diameter. For assembly two holes of 5 in. diameter are cut in the nylon tube 19 in. apart, and stretched to a diameter of 6 in. (by means of a conical flask). The collar so formed fits over the glove supports. The gloves have thickened ends which fit over the nylon collars and are secured in place by Jubilee clips (Plate 3).

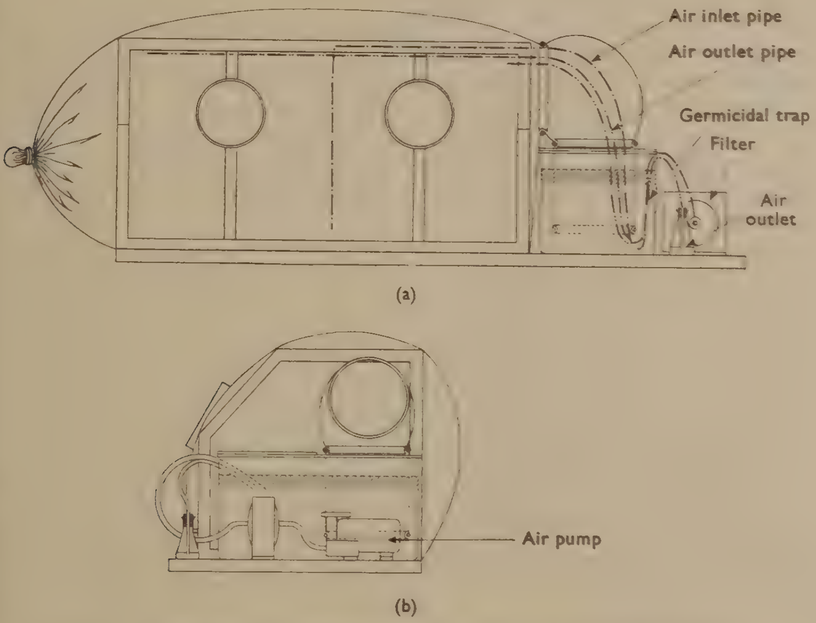

Fig. 1. Longitudinal (a) and lateral (b) sections of an autoclavable plastic germ free unit.

Air is sterilized by passage through a glove box filter (Vokes Ltd., Guildford, Surrey) and fed into the centre of the apparatus by an air pipe which passes through the germicidal trap (Fig. 1). Air is collected at opposite corners through two nylon pipes which pass out via the germicidal trap into a small bottle of germicide (1 in 200 Chloramine T). The apparatus is maintained under positive air pressure which is determined partly by the depth of the outlet tube in the Chloramine T.

Sterilization of the unit. The sterilization procedure is based on that described by Gustafsson (1948). The apparatus is assembled, loaded and placed in the autoclave. The autoclave is fitted with a tap so that germicide can be run into the trap of the unit while the apparatus is still inside, and is also fitted with a small-bore tube enabling sterilized air to pass into the closed autoclave after sterilization.

The autoclave is evacuated to −20 mm Hg, steam is passed in until the pressure reaches 2–3 lb/in² and steaming at this pressure is continued for 10 min. The pressure is raised to 15 lb/in², held for 30 min, and allowed to fall gradually. The germicide (an aqueous solution containing 1,000 p/m of benzalkonium chloride contained in a tank above the autoclave) is then run into the germicidal trap. A sterile glove box filter is then connected to the air pipe leading into the autoclave and sterile air is blown through the filter of the apparatus to dry it. The next day the apparatus is removed from the autoclave and air is blown through the filter to continue the drying. When dry the apparatus is ready for use.

The experimental procedure was as follows. A nylon mat was placed on the floor of the unit after assembly. On to it were placed 2 chick cages (17 in. × 10 in. × 11 in.) modified so that one half of the top could be slid open. Each cage contained a stainless steel feeding trough, a stainless steel water trough, and a stainless steel or glass drinking fountain. A wet and dry bulb thermometer and a tray containing hook and chain for introducing eggs, scissors, tin opener, artery forceps, gauze, and a pair of cloth over-gloves were placed on top of the cages. Forty tins of sterilized canned water (Smedleys Ltd., Whyteleafe, Surrey) or Kilner jars of distilled water were also included.

Operation for rearing germ free chicks. The diet used was the practical chick meal used in previous experiments (Lev & Briggs, 1956). This was packed between two layers of plastic and sterilized by irradiation with γ-rays at 5 Mrads. This dose of irradiation was found to be adequate for sterilization without causing too much destruction of the components of the diet (Ford, Gregory & Thompson, 1959).

Fertile eggs which had been incubated for 18 days were used for the experiments. The procedure used for sterilizing the shells was based on those of Reyniers *et al.* (1949) and Forbes & Park (1959). Six eggs were placed in a net bag and immersed successively in 2% (v/v) Lissapol for 2 min and 2% $HgCl_2$ for 8 min, then rinsed in an aqueous solution of 1,000 p/m benzalkonium chloride and passed into the apparatus through the germicidal trap also containing benzalkonium chloride, by a hook and chain. All solutions except that in the trap were maintained at 37·5° and the eggs were gently manipulated during immersion. Eighteen eggs were introduced for each experiment.

The germicidal trap is not intended to sterilize materials; therefore all materials passed through the trap are previously surface-sterilized. They are wrapped in two layers of nylon before sterilization and the outer layer is removed just prior to immersion, and the inner layer is removed in the germicidal trap. The materials are then allowed to remain in the trap for 30 min before being passed into the chamber. Operators passing material through the trap wear sterile rubber gauntlets or a sterile armlength nylon tube.

After autoclaving and drying, the unit was transferred to a small room at 37° in which a relative humidity of 65% was maintained. When the chicks had hatched eight were selected, if available, and excess chicks were killed and removed. The diet was introduced into the unit and sterility tests begun. The temperature in the room was gradually reduced to 20° over a period of two weeks. Faeces were removed from the cages at weekly intervals.

Assessment of sterility. Sterility checks of the chicks were at weekly intervals. A special stainless steel can containing 4 tubes of trypticase-soy broth, 4 tubes of fluid thioglycollate medium and 2 tubes containing swabs, was introduced into the unit. Vent swabs and samples of the faeces were taken and inoculated into the liquid media inside the unit. Other swabs were streaked outside the unit on to trypticase-soy-blood plates which were incubated aerobically and anaerobically. Potato-dextrose agar and Sabouraud's agar were also inoculated and incubated aerobically for moulds and in addition smears from some of the swabs were examined microscopically after staining by Gram's method.

RESULTS

To date three experiments have been done with this apparatus, two of which were successful in that no bacterial or fungal contamination occurred. In the first experiment, 2 chicks were kept in a germ free condition for a period of 5 weeks, after which they became too large for the cages. In the second, 7 chicks were maintained in a germ free condition for an experimental period of 4 weeks, but in the third, the chicks became contaminated with a Gram-positive sporeforming organism which, it was believed, gained entry into the unit when the eggs were being introduced. The eggs used in this experiment were unavoidably dirty. The germ free chicks reared in the apparatus appeared healthy and of average weight.

DISCUSSION

Nylon is cheap, transparent, withstands autoclaving, and is well suited for use in germ free apparatus. Although the apparatus described has had only a limited number of runs, it has proved to be workable. There are several advantages of a unit made of flexible material compared with rigid apparatus: a positive pressure is always maintained in the apparatus (even when manipulating the gloves), which results in less chance of contamination occurring through a possible leak; lack of rigidity also means that when entry is made into the apparatus the germicide is not displaced from the trap as in the Gustafsson unit; and the layout is designed so that one person, by putting a left hand in the right hand glove, can place materials in the apparatus and remove them with ease.

The apparatus was designed as a prototype, easy modification of which can be made to suit special purposes such as those involving mammals as described in the experiments of Reyniers, Trexler & Ervin (1946), Gustafsson (1948) and Phillips, Wolfe & Gordon (1959).

The nylon tube can be used for at least two experiments and is easily replaced. Experiments are in progress to eliminate the use of a heated room for hatching eggs by fitting the apparatus with a thermostat and employing direct heat. Also, the availability of lay-flat tubing of 40 in. width will greatly facilitate the construction of the apparatus.

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REFERENCES

FORBES, M. & PARK, J. T. (1959). Growth of germ-free and conventional chicks. Effect of diet, dietary penicillin and bacterial environment. *J. Nutr.* **67**, 69.

FORD, J. E., GREGORY, M. E. & THOMPSON, S. Y. (1959). *Rep. nat. Inst. Res. Dairy., Reading*, p. 104.

GUSTAFSSON, B. E. (1948). Germ-free rearing of rats. *Acta path. microbiol. scand.* Supplement no. 73.

GUSTAFSSON, B. E. (1959). Lightweight stainless steel systems for rearing germ free animals. *Ann. N.Y. Acad. Sci.* **78**, 17.

LEV, M. & BRIGGS, C. A. E. (1956). The gut flora of the chick. II. The establishment of the flora. *J. appl. Bact.* **19**, 224.

PHILLIPS, B. P., WOLFE, P. A. & GORDON, H. A. (1959). Studies on rearing the guinea pig germ-free. *Ann. N.Y. Acad. Sci.* **78**, 183.

REYNIERS, J. A. (1959). Design and operation of apparatus for rearing germ free animals. *Ann. N.Y. Acad. Sci.* **78**, 47.

REYNIERS, J. A., TREXLER, P. C. & ERVIN, R. F. (1946). Rearing germ-free albino rats. *Lobund Rep.* no. 1.

REYNIERS, J. A., TREXLER, P. C., ERVIN, R. F., WAGNER, M., LUCKEY, T. D. & GORDON, H. A. (1949). Rearing germ-free chickens. *Lobund Rep.* no. 2.

TREXLER, P. C. & REYNOLDS, L. I. (1957). Flexible film apparatus for the rearing and use of germ-free animals. *Appl. Microbiol.* **6**, 146.

EXPLANATION OF PLATES

Plate 1. Autoclavable plastic unit showing the germicidal trap, air filter and pump.
Plate 2. Autoclavable plastic unit. End view.
Plate 3. Autoclavable plastic unit. General view.

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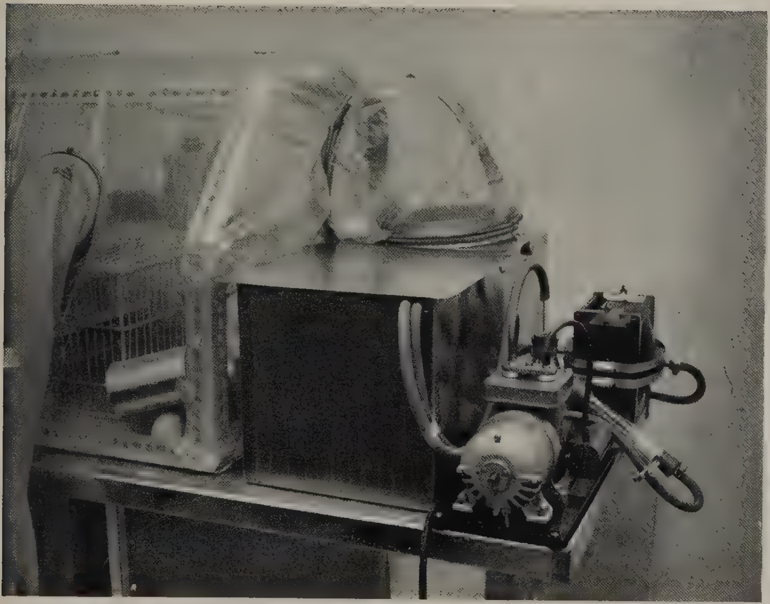
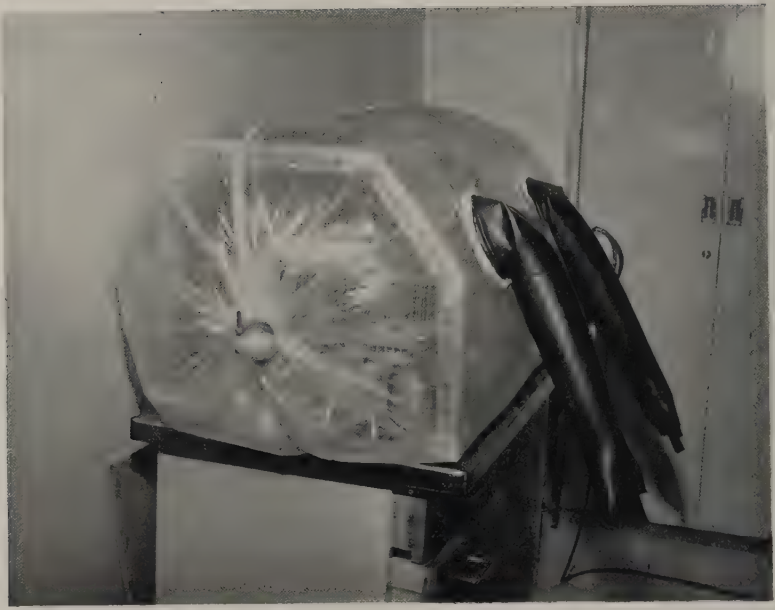
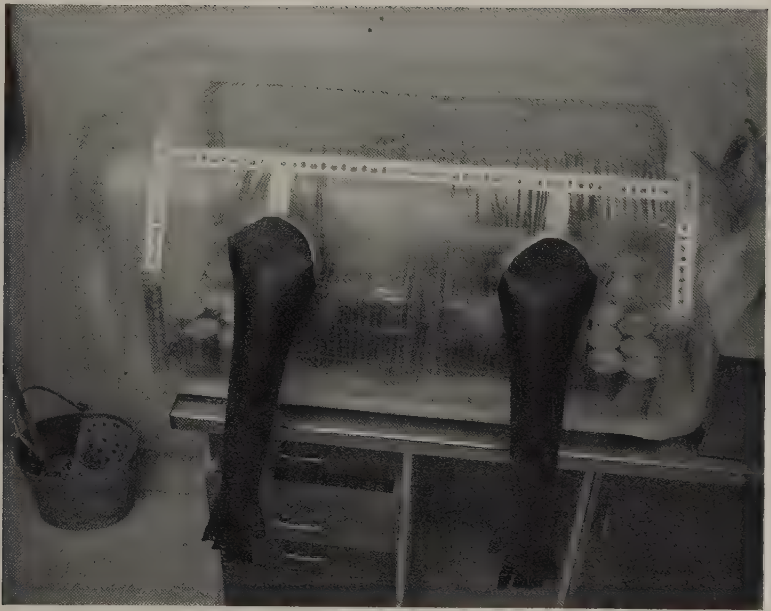


M. LEV — AN AUTOCLAVABLE PLASTIC UNIT FOR REARING ANIMALS UNDER GERM FREE CONDITIONS. PLATE 1



M. LEV — AN AUTOCLAVABLE PLASTIC UNIT FOR REARING ANIMALS UNDER GERM FREE CONDITIONS. PLATE 2



M. LEV — AN AUTOCLAVABLE PLASTIC UNIT FOR REARING ANIMALS UNDER GERM FREE CONDITIONS. PLATE 3

Gould, G. W. (1962). *J. appl. Bact.* **25** (1), 35–41.

MICROSCOPICAL OBSERVATIONS ON THE EMERGENCE OF CELLS OF *BACILLUS* SPP. FROM SPORES UNDER DIFFERENT CULTURAL CONDITIONS

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SUMMARY: Spores of *Bacillus* spp. germinate in suitable environments prior to shedding their outer walls or spore coats. The effects of environmental factors on the way in which spore coats open have been studied. These have included temperature, pH, availability of metals, salt concentration, the presence of coat-lytic enzymes and the effect of preheating the spores.

Generally, spores of large celled species leave behind ill defined structures probably because they dissolve their coats. Spores of small celled organisms leave behind well defined coats after the new vegetative cells have emerged and different strains and species open their coats in a number of different ways. Of the cultural changes, only the pH of the medium markedly affected the mechanism of coat opening. Low pH reduced the extent to which large spores lyzed their coats, and altered the type of coat opening typical of spores of certain small celled species.

In a suitable environment *Bacillus* spores germinate and lose their resistance to heat, appear dark under the phase contrast microscope and become easily stainable while still retaining the shape of heat resistant spores. After this step is completed, the germinated spores swell and shed their coats to allow the young vegetative cells to emerge, elongate and divide. The manner in which the coat opens is often characteristic of the strain, and a number of mechanisms may be seen to occur in different members of the genus *Bacillus*. Lamanna (1940) related the mode of coat opening to the species and stated that when easily visible this character was of taxonomic value. The five main types of emergence described by Lamanna were briefly as follows: small celled species with spore coats opening polarly; small celled species with spore coats opening equatorially by splitting along the transverse axis, by not splitting along the transverse axis or by comma shaped expansion (with half of the coat being carried on each end of the elongated cell); and large celled species opening by absorption (or lysis) of the coat.

The cultures used by Lamanna exhibited little variation with the exception of one culture showing polar emergence which later became absorptive (or lytic). Other workers have noticed that the mechanism of emergence is not stable and varies with the strain, and in some instances even within the strain (Knaysi, 1938, 1957). However the factors responsible for this variation do not appear to have been studied. During an investigation of the effect of inhibitors on spore development it was noticed that the type of spore development often correlated well with the effect of, or resistance to, the inhibitor being tested. This correlation was most marked between the effect of the inhibitor and the way in which the spore coat opened. Since the methods by which the spore coats of *Bacillus* species open are not considered to be stable characters (at least not sufficiently stable for taxonomic use), a study was made to determine the type and extent of any variation induced by changing the environmental conditions during germination and outgrowth.

MATERIALS AND METHODS

Cultures. The organisms used were *B. cereus* NCTC 945, NCTC 8035 and PX; *B. subtilis* NCTC 2590 (syn. *B. aterrimus*) and *B. subtilis* var. *niger* (*B. globigii*); *B. licheniformis* NCTC 7589; *Bacillus* S6, MP5, MP3 and CX. *B. cereus* PX was obtained from R. E. Strange (Microbiological Research Establishment, Porton) and *B. globigii* from W. Bale (M. R. E., Porton). *Bacillus* S6, MP5, MP3 and CX were laboratory isolates chosen because of their methods of emergence and have not been assigned specific names.

Preparation of spore suspensions and extracts. The organisms were grown on potato-glucose agar at 30°. and when sporulation and lysis of the sporangia were complete the growth was recovered in saline at 2° and washed three times with distilled water. These stock suspensions were stored at 2° and normally activated by heating for 30 min at 70° before use. Exosporia were removed from some spore suspensions by ultrasonic vibration using the technique of Berger & Marr (1960). The success of this treatment was judged by examining treated spores mounted in 0·75% (w/v) aqueous crystal violet under a phase contrast microscope. This treatment rendered exosporia readily visible as dark envelopes around refractile spores.

Spore extracts containing lytic enzymes were made by disintegrating spores of one organism in the Mickle tissue disintegrator, then extracting and concentrating the enzyme by the method of Strange & Dark (1957*a*). The organism used was *B. cereus* PX, the strain used by Powell & Strange (1956) and Strange & Dark (1957*a*,*b*) to produce the enzyme which released hexosamine-containing peptides from spore coats and lyzed vegetative cell walls. Activity of the extracts was assessed by their ability to lyze chloroform-treated *E. coli* (Work, 1959) at 45°.

Germination and outgrowth tests. Development of spores was followed microscopically by inoculating slivers of solid medium on 1 in. × 3 in. microscope slides, a similar technique to those used by Lamanna (1940), Pulvertaft (1950) and Pulvertaft & Haynes (1951). After inoculation with one loopful of a suspension of about 10^7 spores/ml, the droplet was dried in a gentle stream of air so that the cells remained immobilized on the agar surface. These microcultures were either incubated, sometimes under coverglasses, in Petri dishes containing strips of damp filter paper or incubated on the microscope stage in a warm chamber.

Germination and outgrowth were studied by periodically examining organisms on the agar surface by phase contrast illumination. This allowed single microcultures to be kept under observation during the progressive stages of germination and outgrowth.

Yeast-glucose agar (YGA) was used as the supporting medium. The normal pH of this medium is 7·2 but in some experiments it was changed by the addition of sterile N HCl or N NaOH to the molten medium just prior to preparing the slides. Other modifications of the test procedure are given under appropriate headings in the Results.

RESULTS

Normal development. Normal development of spores exhibiting the various types of emergence was characteristic and easily recognized. Spores of large celled species dissolved most of their coats after germinating, leaving pale remnants of coats behind (Plate 1, a). Most of this remnant apparently consisted of exosporium which resisted lysis. Spores of *B. cereus* NCTC 945 (which normally had large exosporia), treated by ultrasonic vibration to remove exosporia, discarded only very pale sheaths during emergence, and in some instances no remnant of spore coat could be seen at all (Plate 1, b). Spores of small celled species normally left behind well defined coats that persisted as distinct structures during outgrowth and even after much growth and cell multiplication had occurred. *B. subtilis* NCTC 2590 (syn. *B. aterrimus*), for instance, shed its coat by splitting equatorially along the transverse axis (Plate 1, c), and in many spores of *B. subtilis* var. *niger* (*B. globigii*) separation of the two half coats occurred, thus resembling emergence by comma-shaped expansion. Similarly, distinct coats were shed by *B. licheniformis* NCTC 7589, which emerged from one side of its spore (Plate 1, d), but differed from *B. aterrimus* in that the spores did not open as if the coats were hinged and thus did not leave behind the characteristic coats shaped like figure 3's. Organisms emerging terminally from their spore coats left behind well defined coats that were not dissolved (Plate 1, e).

The effect of temperature. Spores of *B. cereus* NCTC 945 (the coats of which normally lyzed during emergence), *B. licheniformis* NCTC 7589 (which gave equatorial emergence with no splitting of coat) and *Bacillus* S6 (the coats of which opened terminally, but were not lyzed) were placed on slide cultures which were preincubated at 20°, 30°, 37° and 45°, then reincubated at the same temperature. The developing cultures were examined at intervals and the fate of the spore coat noted. Immediately after the spores had opened, their coats (especially those of the lytic type) sometimes adhered to the new vegetative cells and were best seen by sliding a coverglass across the surface of the culture to detach a proportion of the coats. During incubation no difference in the mode of coat opening of any of these spores could be seen, so that temperature does not affect this process. The rate of germination and outgrowth of *B. licheniformis* was extremely slow at 20° and *B. cereus* germinated at 45° but developed no further. Otherwise the spores opened in their characteristic manners between 20° and 45°.

The effect of cobalt and EDTA. Spore lytic enzymes able to attack the spore coat or cell wall were discovered by Powell & Strange (1956), Strange & Dark (1957*a*,*b*) and Work (1959). It is likely that the germinating large spores destroy their coats by lyzing them, because the remaining spore coats have a hazy ill-defined appearance. The enzymes studied by Strange & Dark (1957*a*,*b*) and Work (1959) are activated by Co^{++}, so it was of interest to look for any effect of cobalt or sequestering agents on spore coat opening. It was reasoned that low concentrations of Co^{++} or of other activating metals in the medium might reduce the extent of coat lysis but higher concentrations would increase coat lysis.

Spores of *B. cereus* NCTC 8035 (lytic), *Bacillus* MP5 (not strongly lytic but exhibiting lytic type terminal emergence), *Bacillus* MP3 (terminal emergence and not lytic) and *B. licheniformis* (equatorial emergence without splitting along the transverse axis or hinging of the coat) were therefore incubated in slide cultures of YGA alone or containing 0·06% (w/v) of ethylenediaminetetra-acetic acid (EDTA) or 100 μg of Co^{++} (as $CoCl_2$)/ml. After incubation at 37° to allow development to take place, no change due to the cobalt or EDTA was apparent. The EDTA allowed germination to occur, but reduced the rates of outgrowth considerably, completely inhibiting *B. cereus* NCTC 8035 and *Bacillus* MP3. This concentration of EDTA allowed *B. licheniformis* and *Bacillus* MP5 to shed their coats normally, but prevented the subsequent elongation and division of cells.

The effect of spore lytic enzymes. Spore suspensions were mixed with an equal volume of the insoluble extract containing lytic enzyme obtained from *B. cereus* PX at pH 3, then inoculated on slide cultures. The extract was active in lyzing cells of chloroform-treated *E. coli* at a pH optimum of 8. Six strains representing the various types of coat opening were used, and included the *B. cereus* PX spores from which the lytic solution was prepared. Development of all the organisms proceeded normally whether the enzyme was present or not, and no increased lysis of any of the spore coats was noted.

The effect of pH of the medium. When spores were inoculated on to YGA adjusted to pH 5·55, 5·8, 6·35, 6·9, 7·35 and 7·95 they germinated and began to develop at different rates. Media adjusted to less than pH 5·55 were not used because germination was slow and incomplete, although spores of some organisms will begin to germinate even at pH 4 (Knaysi, 1945). Spore coats of *B. licheniformis* opened and appeared similarly shaped and distinct at all pH values, but for some organisms the effect of pH on the mechanism of coat opening was quite marked.

Spores of *B. cereus* NCTC 8035 left only pale remnants of coats after development at pH 7·0 or above, but at pH 5·55 or 5·8 the extent of lysis of the coat was much less pronounced (Plate 2, a and b). At these low pH values germination and emergence of vegetative cells from the spores occurred rapidly although cell growth and multiplication were retarded. With the less strongly lytic *Bacillus* MP5, this effect of pH was more marked. At pH 5·55 the discarded spore coats remained very distinct whereas above 6·9 much dissolution of the coat material occurred.

Discarded spore coats of *Bacillus* CX were well defined at both extremes of pH, but the method by which the coats opened changed at different pH values. At pH 6·9 and above coats opened equatorially by splitting along the transverse axis (Plate 2, c) so that empty coats shaped like figure 3's were discarded by the elongating vegetative cells. At the lower pH values, however, a different mechanism was seen to operate. Instead of remaining hinged together the two half coats separated and remained attached to each end of the elongating vegetative cell. At pH 5·55, in particular, all the spores opened in this manner, and a high proportion of the new vegetative organisms curved as they elongated, forming horseshoe shaped cells with half-coats on each end (Plate 2, d). At pH 5·8 this was still the predominant method of spore coat opening whereas at pH 6·35 the majority of spores in a population opened by the usual hinged method. Thus a change in mechanism was induced by low pH.

The effect of salt concentration. The same four types of coat opening were compared in the presence of 0, 1, 3 and 6% (w/v) sodium chloride. Germination and emergence occurred in all cases and apart from a reduced rate of outgrowth in the higher salt concentrations and a tendency for the new vegetative cells to become curved, the presence of salt had no effect.

The effect of heat activation. Spores exhibiting the various types of coat opening were heated in water at 80° for 30 min before being incubated at 30° on slide cultures. Their development was compared with that of the same spore stocks which had been stored for over one month at 2° in water and had not been heated. This excessive heat activation had no effect on the way in which spore coats opened except that about 5% of heated *B. cereus* NCTC 8035 spores became dark under the phase contrast microscope and failed to develop.

DISCUSSION

Variation in the mechanism of emergence from the spore shown even by the same strain of *Bacillus* species has been well substantiated. This is particularly apparent for *B. megaterium* strains which, although described by Lamanna (1940) as absorptive, were shown by Levinson & Wrigley (1960) to split their coats along well defined equatorial ridges or grooves, leaving behind hinged half-coats shaped like figure 3's. On the other hand, the *B. megaterium* strains studied by Bradley & Franklin (1958) had smooth or slightly ribbed spores, and the *B. megaterium* spores used by Robinow (1945) shed pale remnants of coats without any hinging being apparent. Differences in the thickness, structure and phosphorus content of the spore coats of *B. megaterium* strains have been noted by Fitz-James & Young (1959) and may be related to the different types of emergence.

B. subtilis has generally been described as displaying the hinged coat type of opening (e.g. Lamanna, 1940; Pearce & Powell, 1951; Harris, 1955; Bartholomew & Clare, 1955, 1956). However, the photographs published by Harris (1955) show empty spore sacs of *B. globigii* which are pale and weak and ruptured at one pole; our strain of *B. globigii* shed well defined coats following equatorial splitting along the transverse axis.

That cells of the same species can emerge from the spore coat at different times in different ways suggests that all spores might open by the action of a lytic enzyme. Spores produced under different conditions or at different times might contain more or less of the enzyme so that the appearance of the empty spore coats reflects the amount of lysis they have undergone. Alternatively, the lytic system might act only on certain regions of the spore coat that contain its substrate in a suitable state for enzymic action. In spores which appear to split their coats this region would be only a small part of the whole, whereas in spores that dissolve most of their coat material away the whole of the coat would contain the enzymes' substrate. The latter alternative seems more likely since discarded coats of small spores, which appear to open by splitting, are stable structures that remain for long periods without obviously lyzing. Of course, the splitting might be solely mechanical and not involve lysis at all; however, electron micrographs of spore coats shed by *B. subtilis* show a translucent area on one side of the coat which is suggestive of lysis rather than mechanical rupture (Hachisuka *et al.* 1955).

It does not appear that any such regions susceptible to lysis or mechanical rupture are necessarily related to the surface configuration of the spore. Spores of varieties of *B. subtilis* and *B. coagulans*, for instance (Franklin & Bradley, 1957; Bradley & Franklin, 1958), often have longitudinal ribs, but rupture their coats equatorially. On the other hand, although *B. megaterium* spores may not always have longitudinal grooves, it appears that when they do the surface structure and the type of coat opening are related (Levinson & Wrigley, 1960) and the normal equatorial emergence of *B. licheniformis* from its spores during outgrowth may well reflect the single longitudinal groove in the spore coat where the wall is thin. Powell (1957) has shown that intact spores are unaffected by the lytic enzymes, so it is to be expected that any region of the coat susceptible to lysis will be beneath the spore surface and therefore need not be noticeable in the spore topography.

Although it is clear that the mechanism of coat opening is not necessarily specific, as it may vary with or within the strain and may change with the passage of time, the results reported in the present work indicate that, using the same stocks of spores, environmentally induced variation is not easily demonstrable. Indeed of all the factors studied here only pH produced any change in pattern, and then not of a very profound nature. The change in the type of splitting of the coat, from hinged coats at neutral pH to complete separation of the two half-coats at lower pH values, cannot be considered to be a very significant variation because some organisms often show a mixture of these two methods at nearly neutral pH (e.g. our *B. globigii* spores at pH 6·9). Although low pH reduces the extent of lysis of coats of lytic spores, they are still recognizably different from the nonlytic spores which open terminally and leave distinct coats after emergence. It is therefore likely that the type of coat opening shown by spores of a single suspension is stable within the limits reported here, and unlikely to change markedly in different environments.

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REFERENCES

BARTHOLOMEW, J. W. & CLARE, P. (1955). Parasporal bodies of *Bacillus* spores. *Bact. Proc.* p. 38.

BARTHOLOMEW, J. W. & CLARE, P. (1956). Parasporal bodies of *Bacillus* spores. *J. Bact.* **71**, 158.

BERGER, J. A. & MARR, A. G. (1960). Sonic disruption of spores of *Bacillus cereus*. *J. gen. Microbiol.* **22**, 147.

BRADLEY, D. E. & FRANKLIN, J. G. (1958). Electron microscope survey of the surface configuration of spores of the genus *Bacillus*. *J. Bact.* **76**, 618.

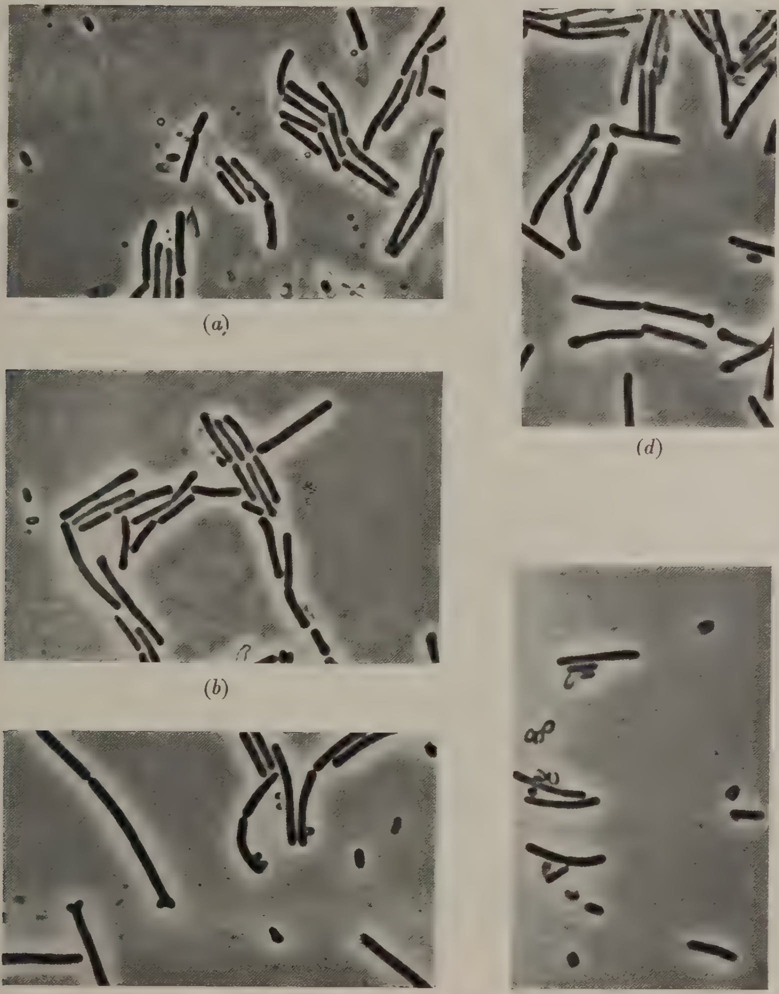
FITZ-JAMES, P. C. & YOUNG, I. E. (1959). Cytological comparisons of spores of different strains of *Bacillus megaterium*. *J. Bact.* **78**, 755.

FRANKLIN, J. G. & BRADLEY, D. E. (1957). A further study of the spores of species of the genus *Bacillus* in the electron microscope using carbon replicas, and some preliminary observations on *Clostridium welchii*. *J. appl. Bact.* **20**, 467.

HACHISUKA, Y., ASANO, N., KATO, N., OKAJIMA, M., KITAORI, M. & KUNO, T. (1955). Studies on spore germination. I. Effect of nitrogen sources on spore germination. *J. Bact.* **69**, 399.

HARRIS, W. J. (1955). Demonstration of the existence of spore sacs around resting spores of *Bacillus subtilis* and *Bacillus globigii*. *J. gen. Microbiol.* **13**, 103.

KNAYSI, G. (1938). Cytology of bacteria. *Bot. Rev.* **4**, 83.



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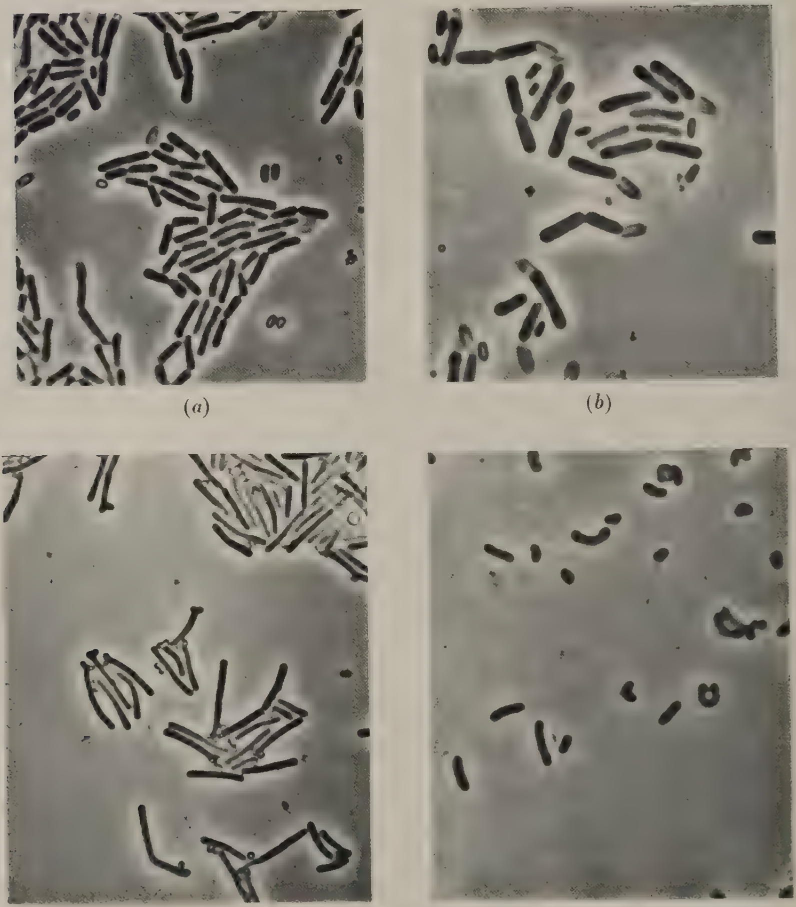
G. W. Gould — Microscopical Observations on the Emergence of Cells of *Bacillus* Spp. from Spores under Different Cultural Conditions. Plate 1



(*a*) (*b*)

(*c*) (*d*)

G. W. Gould — Microscopical Observations on the Emergence of Cells of *Bacillus* Spp. from Spores under Different Cultural Conditions. Plate 2

KNAYSI, G. (1945). Investigation of the existence and nature of reserve material in the endospore of a strain of *Bacillus mycoides* by an indirect method. *J. Bact.* **49**, 617.

KNAYSI, G. (1957). Structure of the endospore and the cytological processes involved in its formation and germination, with remarks on the criteria of germination. *J. appl. Bact.* **20**, 425.

LAMANNA, C. (1940). The taxonomy of the genus *Bacillus*. I. Modes of spore germination. *J. Bact.* **40**, 347.

LEVINSON, H. S. & WRIGLEY, A. S. (1960). Spore germination and emergence of *Bacillus megaterium*. *Science* **131**, 1382.

PEARCE, T. W. & POWELL, E. O. (1951). New techniques for the study of growing micro-organisms. *J. gen. Microbiol.* **5**, 91.

POWELL, J. F. (1957). Biochemical changes occurring during spore germination in *Bacillus* species. *J. appl. Bact.* **20**, 349.

POWELL, J. F. & STRANGE, R. E. (1956). Biochemical changes occurring during sporulation in *Bacillus* species. *Biochem. J.* **63**, 661.

PULVERTAFT, R. J. V. (1950). The formation and germination of bacterial spores studied by phase contrast and slide cell culture. *J. gen. Microbiol.* **4**, xiv.

PULVERTAFT, R. J. V. & HAYNES, J. A. (1951). Adenosine and spore germination; phase-contrast studies. *J. gen. Microbiol.* **5**, 657.

ROBINOW, C. F. (1945). In addendum to *The Bacterial Cell*, by R. J. Dubos. Cambridge. Massachusetts: Harvard University Press.

STRANGE. R. E. & DARK, F. A. (1957*a*). A cell-wall lytic enzyme associated with spores of *Bacillus* species. *J. gen. Microbiol.* **16**, 236.

STRANGE, R. E. & DARK, F. A. (1957*b*). Cell-wall lytic enzymes at sporulation and spore germination in *Bacillus* species. *J. gen. Microbiol.* **17**, 525.

WORK, E. (1959). The action of a lytic enzyme from spores of a *Bacillus* on various species of bacteria. *Ann. Inst. Pasteur* **96**. 468.

EXPLANATION OF PLATES

PLATE 1. Phase contrast micrographs of developing *Bacillus* spores, showing open spore coats and emerging vegetative cells (×1,540).

(a) *B. cereus* NCTC 945. Discarded spore coats are pale and almost completely lyzed; exosporia are visible as pale sheaths.
(b) *B. cereus* NCTC 945. Exosporia were removed from the spores before incubation; very little of the true spore coat has escaped lysis.
(c) *B. aterrimus* NCTC 2590. Equatorial emergence with splitting of the spore coat.
(d) *B. licheniformis* NCTC 7589. Equatorial emergence without splitting of the spore coat.
(e) *Bacillus* S6. Polar emergence.

PLATE 2. Phase contrast micrographs of developing *Bacillus* spores at different pH values (×1,540).

(a) *B. cereus* NCTC 8035 at pH 6·9; extensive lysis of spore coats has occurred.
(b) *B. cereus* NCTC 8035 at pH 5·55; spore coats have not been completely lyzed.
(c) *Bacillus* CX at pH 6·9; spore coats have opened but the half-coats remain connected.
(d) *Bacillus* CX at pH 5·55; after opening, the half-coats have separated.

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Andrla, O. & Briggs, C. A. E. (1962). *J. appl. Bact.* **25** (1), 42–45.

THE INCIDENCE OF LECITHINASE POSITIVE *CLOSTRIDIUM WELCHII* IN THE PIG AND THE EFFECTS THEREON OF ANTIBIOTIC FEED SUPPLEMENTS

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SUMMARY: The varying incidence of *Clostridium welchii* in the gut of growing pigs, and the suppression of these organisms on addition to the diet of nutritional levels (50 g/ton) of antibiotics, have been demonstrated. The degree of lecithinase activity of the organisms and administration of antibiotics were correlated. Growth stimulation was not demonstrable in older pigs, though antibiotic feed supplements may have some prophylactic value for such animals.

It is well known that the growth of young animals and humans is improved by the addition of antibiotics to their diet. Though there are several opinions concerning the mode of action of antibiotics, their effect is generally attributed to their bacteriostatic action.

Following the work of Lev, Briggs & Coates (1957), later confirmed by Williams Smith (1959), it seems that one of the factors in the growth stimulating effect of antibiotics in the chick is the suppression of the multiplication of lecithinase (α-toxin) producing *Clostridium welchii*; further, that the lecithinase activity of *Cl. welchii* in the gut of the chick may be impaired by administering dietary antibiotics and subsequently restored by transferring the organisms into a suitableantibiotic free medium.

Since antibiotic feed supplementation is as important in the pig industry as it is in poultry production, our investigations were intended to clarify the role of lecithinase positive *Cl. welchii* in the gut flora of the pig and the effect of antibiotics thereon.

Methods

Eighteen crossbred pigs (Essex × Large White), consisting of two litters of nine, were used; they were weaned at 9 weeks of age, had never received antibiotics and came from non-antibiotic-fed stock; they were weighed weekly and faecal samples were collected from each pig once a week from the 12th to the 42nd week of age. At 34 weeks of age twelve of the eighteen pigs were selected according to weight and divided into four groups of three, so that the intergroup weight variation was of the order of ±0·2%. Because of unavoidable circumstances the pigs were not weighed on the 33rd or 34th week, so that the weight bases used for arranging the groups and for subsequent calculation of weight gains were the figures for the 32nd week weighing: likewise, the pigs were last weighed at the 41st week of age. From the

34th week, groups A, P and T received chlortetracycline, penicillin G or oxytetracycline, respectively, at 50 g/ton whereas group C remained on the unsupplemented control ration. One pig in group T had to be destroyed in the 35th week owing to injury.

Modified Nagler's medium containing 30% of fresh human serum (the same batch throughout) and neomycin sulphate (Mycifradin, Upjohn) at 100 μg/ml was used to determine colony counts of *Cl. welchii* and for the detection of lecithinase activity. The plates (5½ in. diam., each containing 50 ml of medium) were incubated anaerobically at 37° for 24 hr and lecithinase activity determined by measuring the zones of lipid precipitation. Anaerobic streptococci also grew on this medium, but these were readily distinguished from *Cl. welchii* by their morphological appearance. Robertson's meat broth was used for subcultivation, and sensitivity to antibiotics was determined by the disc method. The chlortetracycline, penicillin and oxytetracycline sensitivity disc concentrations were 5 μg, 2 i.u. and 5μg, respectively.

RESULTS

Although the control, unsupplemented diet was given to all the pigs from 9 to 33 weeks of age, the numbers of *Cl. welchii* in their faeces declined steadily over the period from 12 to 15 weeks until, at 15 weeks, none were isolated (Table 1). It was noted also that, prior to 15 weeks of age, as the numbers of *Cl. welchii* declined, the lecithinase activity of each colony gradually diminished. The reverse process occurred after 30 weeks of age when, as the numbers of *Cl. welchii* were increasing, lecithinase activity was gradually restored (Plate 1, a).

Table 1. *The incidence of* Cl. welchii *in the faeces of pigs receiving unsupplemented and antibiotic supplemented diets*

Pig no.	Nos. (10^3/g wet wt.) in faeces of pigs on unsupplemented diet, aged (weeks)							Supplement* given from 34th week	Nos. (10^3/g wet wt.) in faeces of pigs on supplemented or unsupplemented diets, aged (weeks)								
	12	13	14	15–30	31	32	33		34	35	36	37	38	39	40	41	42
1	3	—†	—	—	0·08	1·5	180	C	5	4000	10	2000	1100	50	4	600	10
2	0·1	—	0·1	—	0·5	1·7	100										
3	0·4	—	—	—	0·1	2	60										
4	10	—	—	—	0·05	0·7	220	T	5	10	500	200	5	—	0·05	0·02	—
5	3	8	—	—	0·065	0·8	20										
6	0·3	—	—	—	0·075	0·6	50	P	7	5	0·15	2	0·2	0·35	0·5	0·4	0·45
7	40	4	—	—	0·085	1·2	500	C	4	5	30	2000	7000	100	4	500	10
8	40	0·5	—	—	4	1·2	170	C	8	8	20	4000	5000	600	5	50	2
9	0·2	—	—	—	1·2	100	1700										
10	0·15	—	—	—	6	80	900	T	4	8	500	20	0·01	0·05	0·02	—	—
11	10	—	—	—	0·8	90	2800	A	30	—	—	2	0·07	0·45	0·45	0·09	—
12	0·2	—	—	—	0·5	80	100										
13	0·1	—	0·1	—	10	100	2000	A	300	5	90	100	0·02	0·16	30	20	0·02
14	2	0·6	—	—	2	200	140	T	4	‡							
15	30	5	3	—	1·5	300	160	P	8	0·5	—	4	30	0·30	0·35	—	—
16	2	—	—	—	5	100	60										
17	4	8	—	—	8	100	800	A	400	10	—	2	—	0·05	0·02	0·02	0·04
18	0·4	—	—	—	1·5	100	70	P	600	70	—	2	—	0·07	0·02	0·23	—

* Supplement: C, none (control, unsupplemented diet throughout); A, chlortetracycline (Aureomycin, Lederle); P, penicillin G; T, oxytetracycline (Terramycin, Pfizer). † —, none isolated. ‡ Injured, destroyed.

The fact that *Cl. welchii* was absent from the faeces of pigs between 15 and 30 weeks of age suggests that natural antibiosis may be functional during this period and no antibiotics were therefore given until 34 weeks of age, when large numbers of *Cl. welchii* were again present and lecithinase activity was well established.

Table 2. *The growth of pigs receiving unsupplemented and antibiotic supplemented diets*

Pig no.	Weights (lb) of pigs, diet unsupplemented, aged (weeks)*						Supplement† given from 34th week	Weights (lb) of pigs, diet supplemented or unsupplemented, aged (weeks)						Mean weight gains from 32nd–41st weeks
	12	13	14	15	30	32		35	36	38	39	40	41	
1	55	62	70	75	220	237	C	257	268	272	286	286	298	
2	60	66	72	77	198	215								
3	62	67	77	89	254	277								Group C, pigs 1, 7, 8: 60 lb
4	66	73	78	88	261	279	T	306	312	320	334	336	346	
5	47	55	64	72	218	220								
6	67	76	85	95	246	260	P	281	280	310	318	330	324	
7	70	78	83	90	260	280	C	300	310	332	326	334	334	Group T, pigs 4, 10, 14: 75 lb
8	67	74	79	86	250	268	C	291	300	314	322	326	334	
9	59	64	70	78	234	251								
10	48	54	58	64	218	244	T	270	287	293	310	318	328	
11	45	52	59	63	222	238	A	250	258	274	282	292	302	Group P, pigs 6, 15, 18: 61 lb
12	41	46	53	58	171	184								
13	60	70	81	89	260	281	A	308	305	325	334	340	346	
14	74	81	85	96	233	259	T	‡						
15	71	81	90	102	268	282	P	297	310	316	328	332	338	Group A, pigs 11, 13, 17: 67 lb
16	61	67	75	82	214	230								
17	65	71	77	83	248	263	A	291	303	298	328	316	336	
18	63	70	77	86	222	243	P	253	275	273	292	288	306	

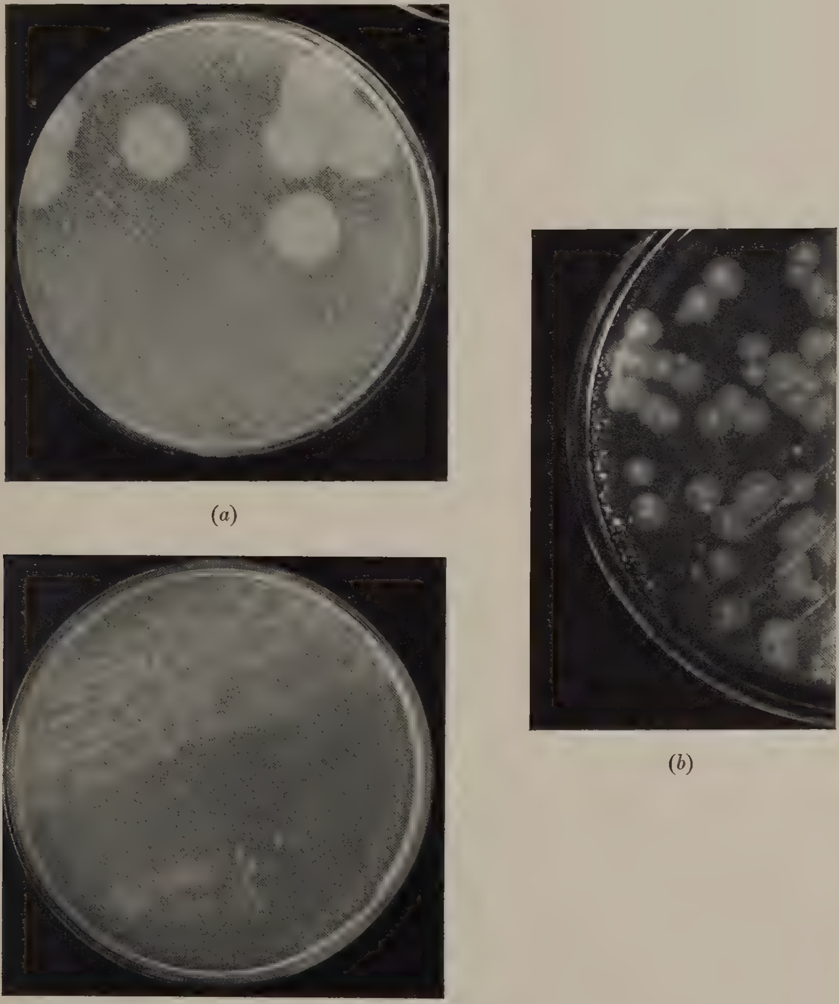
* No weighing at 31, 33, 34, 37 and 42 weeks of age. † Supplements, all at 50 g/ton of feed: C, none (control, unsupplemented diet throughout); A, chlortetracycline (Aureomycin, Lederle); P, penicillin G; T, oxytetracycline (Terramycin, Pfizer). ‡ Injured, destroyed.

Antibiotic supplementation of the diet caused a sharp decline in the numbers of *Cl. welchii* by comparison with the control group (Table 1). Their complete elimination did not result in every case at the antibiotic level used and the counts fluctuated to some degree. However, lecithinase production declined and ultimately ceased completely (Plate 1, b and c), though this could not be correlated with any significant weight gains (Table 2). Surviving atoxigenic organisms were not found to be resistant to any of the three antibiotics used. The ability to produce lecithinase was resumed after subcultivation in antibiotic free media and, in fact, on primary isolation after discontinuing the antibiotic feed supplements after 42 weeks.

Discussion

It thus appears that antibiotics in the gut of the pig act on *Cl. welchii* in a manner similar to that previously described in the chick. *Cl. welchii* is often implicated as a cause of disease in animals and man; it is therefore of considerable importance to differentiate between true nonproducers of lecithinase and those strains that appear, from the effects of antibiotic feeding or therapy, to be lecithinase negative. Although, not unexpectedly, no growth improvement was occasioned in older pigs by the administration of the antibiotic supplements, their effects on the numbers and lecithinase activity of *Cl. welchii* indicate some potential prophylactic value in the use of antibiotic feed supplements for such animals. The possibility of natural antibiosis in the gut of the pig between 15 and 30 weeks of age is supported by the finding of similarly impaired lecithinase activity of *Cl. welchii* following the feeding of antibiotic supplements.

It is evident that the effect of dietary antibiotics in depressing α-toxin production of *Cl. welchii* in the gut is similar in both the pig and the chick; but that in the older pig, unlike the chick, there is no concomitant improvement in growth rate.



(*a*)

(*b*)

(*c*)

O. Andrla & C. A. E. Briggs — The Incidence of Lecithinase Positive *Clostridium welchii* in the Pig and the Effects Thereon of Antibiotic Feed Supplements. Plate 1

We wish to thank Miss S. Bradley for technical assistance, and Mr. A. L. Warren and the Animal Technicians of this Department for help with the experimental animals.

REFERENCES

LEV, M., BRIGGS, C. A. E. & COATES, M. E. (1957). The gut flora of the chick. 3. Differences in caecal flora between 'infected', 'uninfected' and penicillin-fed chicks. *Brit. J. Nutr.* **11**, 364.

SMITH, H. WILLIAMS (1959). The effect of the continuous administration of diets containing tetracyclines and penicillin on the number of drug-resistant and drug-sensitive *Clostridium welchii* in the faeces of pigs and chickens. *J. Path. Bact.* **77**, 79.

EXPLANATION OF PLATE

Lecithinase production on modified Nagler's medium of *Cl. welchii* isolated from the faeces of pig no. 4.

(a) At 33 weeks of age, before receiving antibiotic supplemented diet: lecithinase activity marked (zone diameter approx. 20 mm); × ½ approx.

(b) At 38 weeks of age, after receiving antibiotic supplemented diet for 5 weeks: lecithinase activity reduced (zone diameter approx. 8 mm); × ⅔ approx.

(c) At 41 weeks of age, after receiving antibiotic supplemented diet for 8 weeks: no lecithinase activity; × ½ approx.

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A SIMPLIFIED METHOD FOR THE MEASUREMENT OF BACTERIAL SURFACE CONTAMINATION IN FOOD PLANTS AND ITS USE IN THE EVALUATION OF PRESSURE CLEANERS

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SUMMARY: A new rapid method 1 for determining microbial surface contamination has been developed. Samples are taken with cotton swabs and transferred directly to agar slants. The slants are grouped according to the number of colonies formed during incubation, and the average number of colonies and its standard deviation are determined by plotting the distribution on probability paper. The method, which is particularly well suited for large scale field examinations, was used to evaluate the efficiency of hydraulic pressure cleaners in a pig slaughterhouse. As a result of the daily use of the pressure cleaners, the bacterial surface contamination on the machinery and equipment was in no place higher than about 100 cm^2 after cleaning. Further, there was a 90% reduction of the surface counts on the dressed pig carcases. Also, the cleaning was more rapidly done than by the traditional methods.

MICROBIOLOGISTS have been concerned with the problem of measuring surface contamination almost as long as bacteria have been known. Much work in this field has been concentrated on the technique by which bacteria are transferred from a solid surface to the medium in which they are counted. Many of the methods developed have been evaluated on the basis of their ability to produce the highest counts possible. For practical purposes, however, it seems more important to obtain a figure which gives a reliable indication of the average contamination of the whole surface in question, rather than the highest possible recovery from a few samples (Silliker, Andrews & Murphy, 1957).

The bacterial population on a contaminated surface generally shows a distribution which is not Gaussian. The numbers of bacteria in individual samples differ greatly, and as a result the averages for small groups of samples taken from the same source do not agree well. This suggests that the arithmetic mean is not suitable for the evaluation of surface contamination. Empey & Scott (1939) found that the distribution of the numbers of bacteria on the surfaces of equipment, meat carcases etc. was approximately log normal, and if this were generally true it would be better to express surface contamination by the logarithms of the counts rather than the counts themselves, one of the advantages being that all the usual statistical tests are done very easily. We have been able to confirm the observations of Empey & Scott and have made use of them to devise a relatively simple method for the evaluation of surface contamination in slaughterhouses. It is believed that the principle of this method can find wider applications.

MATERIALS AND METHODS

Pressure cleaners

Installation of pressure cleaners. The pressure cleaners (Sellers Injector Corp., Philadelphia, U.S.A.), which operated on the steam injection principle and had no moving parts, and could be adjusted by changing the spray nozzles and venturi tubes, were installed in various places in a slaughterhouse. A high pressure jet cleaner (type B) with a capacity of 1,000 gal/hr was installed on the killing floor and used to clean the dehairing and scraping machines and the eviscerating table. A similar cleaner was installed on the hanging floor, where it was used mainly for cleaning wooden boxes, carts and cutting tables. In the sausage plant a Booster Jet Cleaner with a capacity of 600 gal/hr was installed. This is a low pressure unit which can be fed with steam down to a pressure of 0·35 atm, whereas the high pressure cleaners need a pressure of at least 2·8 atm.

Efficiency of pressure cleaning equipment. The pressure cleaners were tested for efficiency by carrying out bacteriological counts on the slaughterhouse equipment before and after cleaning. The cleaners were installed in a medium sized pig slaughterhouse (handling approximately 120,000 pigs annually) and the effects of the cleaning were determined by daily bacteriological examinations, using the methods described below. The effects of the cleaning were also determined by making daily examinations of dressed pig carcases, samples being taken from the rind side of the first and last ten pigs slaughtered every working day during the 7 weeks of investigations.

Bacteriological examinations commenced one week before the cleaners were installed, in order to assess the existing level of sanitation.

Rapid assessment of surface contamination

The differences between means of surface counts are normally considerable, irrespective of the technique used for sampling and counting. In order to obtain useful results it is necessary to reduce the variation to a reasonable level, and the only way to do this is to increase the number of samples considerably. However, this is only possible if a simplified technique for sampling and counting can be used. To this end the following technique was developed.

Sampling. Ten or more samples were taken from each surface (piece of equipment or carcase) in the following way. A sterile cotton swab was moistened with sterile tap water containing 4% (w/v) of NaCl, and a 10 cm^2 area of the surface, demarcated by a metal frame, was swabbed vigorously without rotating the swab. The bacteria removed were transferred to the surface of an agar slant by wiping the dirty side of the swab in a zigzag line from the bottom of the slope to the top. Not all of the bacteria on the sampled area are removed by the swab, and of those removed only a fraction are deposited on the slant. This, however, is not a serious problem as long as good reproducibility is obtained, as discussed later.

Culture medium. In order that the agar slopes should have a sufficiently hard surface the following medium was used: Oxoid Blood Agar Base containing 3·5% of NaCl and reinforced with 0·5% of agar (Difco).

This medium has been used during 3 years' work in 70 Danish pig slaughterhouses concerned mainly with the production of Wiltshire bacon. The NaCl was added because there seems to be a close correlation between counts on this medium, incubated for 4–5 days at 20°, and the storage life of bacon.

Table 1. *The results of the examination of a tiled wall in a pig slaughterhouse, using 10 slants*

Range of nos. of colonies	No. of slants	Cumulative frequency (%)	
		Observed	Corrected
0–3	0	0	—
3–10	1	10	5
10–30	1	20	15
30–100	2	40	30
100–300	2	60	50
300–1,000	3	90	75
>1,000	1	100	—

Colony counting. Counting of all the colonies which developed on the slants was avoided, the slants being put into groups based on counts in the ranges 0–3; 3–10; 10–30; 30–100; 100–300; 300–1,000; and >1,000. These groups are convenient as they each cover approximately half a $\log_{10}$ cycle.

After a short period of training it is possible for a technician to do this grouping by visual inspection without actually counting the colonies on the slant.

Calculation of the logarithmic means. From each surface examined the numbers of samples in the different groups are obtained, and the results plotted on probability paper as a grouped distribution, which is corrected by a method similar to that suggested by Bliss (1937).

The results of the examination of a tiled wall in a pig slaughterhouse may be used to illustrate the method (Table 1).

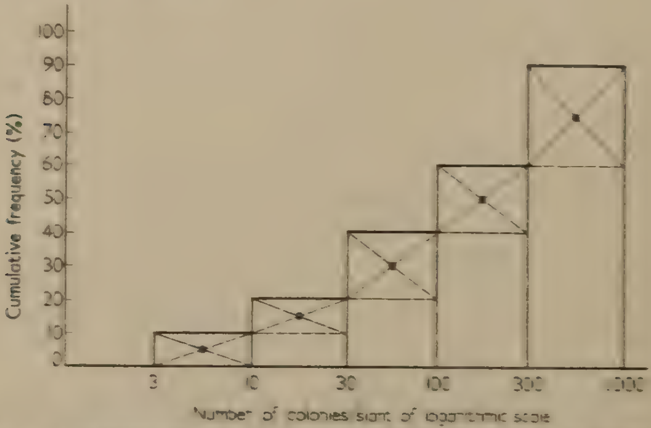

Fig. 1. Histogram showing the cumulative frequencies of ranges of colony count obtained from 10 samples, with the 'corrected' values (open circles).

The cumulative frequencies are shown in Fig. 1 as a histogram. The 'corrected' cumulative frequencies are then obtained by marking the mid points of the projections of each column above the one on its left, as shown by the open circles in Fig. 1. The values of these 'corrected' cumulative frequencies appear in the last column of Table 1. The 'corrected' cumulative frequencies are then plotted on probability paper against the mean value of the range of count for the corresponding group, as shown in Fig. 2, and the logarithmic mean of the distribution is derived from the straight line passing through the points, being the value for a frequency of 50%. The standard deviation of the distribution is represented by the difference between the values for frequencies of 50% and 16%.

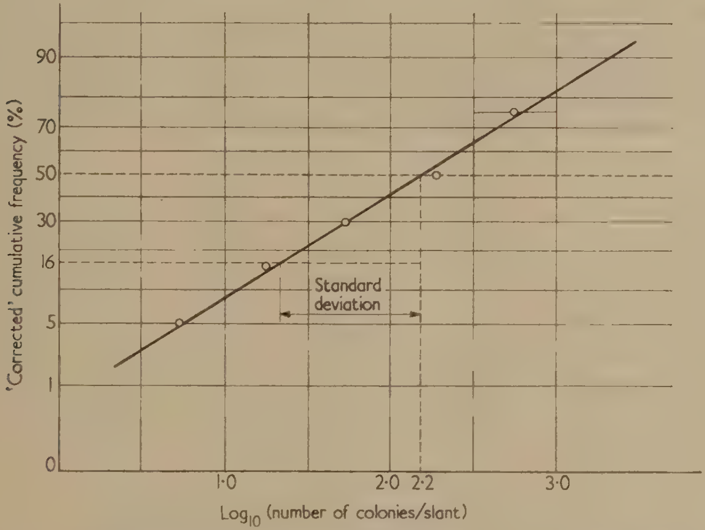

Fig. 2. The determination of the logarithmic mean of the 'corrected' distribution of the colonies on a series of agar slants, using the data of Table 1 and probability paper.

The standard deviation is normally of the order of 0·5–1·0 log cycles and its value does not seem to depend on the method used for sampling or counting. The primitive but rapid method used for transferring the sample from equipment to the agar slants results in counted which were 1·0–1·2 log cycles lower than those obtained by traditional sampling and plating procedures. However, the difference seems to be constant when the counts are not too high. They can be corrected if desired.

Results and Discussion

Cleaning of the scraping machine. Before the pressure cleaner was installed and during the first days after it was put into use the average bacterial counts on the blades of the cleaned machine exceeded $10^4/\text{cm}^2$. After daily cleaning with the pressure cleaner the number of surface bacteria was brought down to between 10 and 100/cm².

Cleaning of the eviscerating table. At the beginning the counts exceeded 10⁴/cm²; afterwards, the average number of bacteria varied between 30 and 200/cm².

Cleaning of a tiled wall. At the beginning the counts were about 300/cm², but during the following 3 weeks the number of bacteria was reduced to less than 10/cm².

Cleaning of the precarving trestles. The use of the pressure cleaner reduced the number of bacteria from 360/cm² to between 10 and 100/cm².

Cleaning of pig carcases. Bacterial counts were obtained from the surface of the first 10 pigs slaughtered every day for a period of 6 weeks. The results of these examinations are shown in Fig. 3. The pressure cleaner was brought into use at the beginning of the second week; however, no great effect of the pressure cleaner was noted either on the pigs or on the equipment during the first 7 days of use. No doubt the reason for this was that the temperature at which the cleaner worked was not fully controlled. In the third week the temperature of the water ejected from the pressure cleaner was controlled; this temperature should be 90–95°.

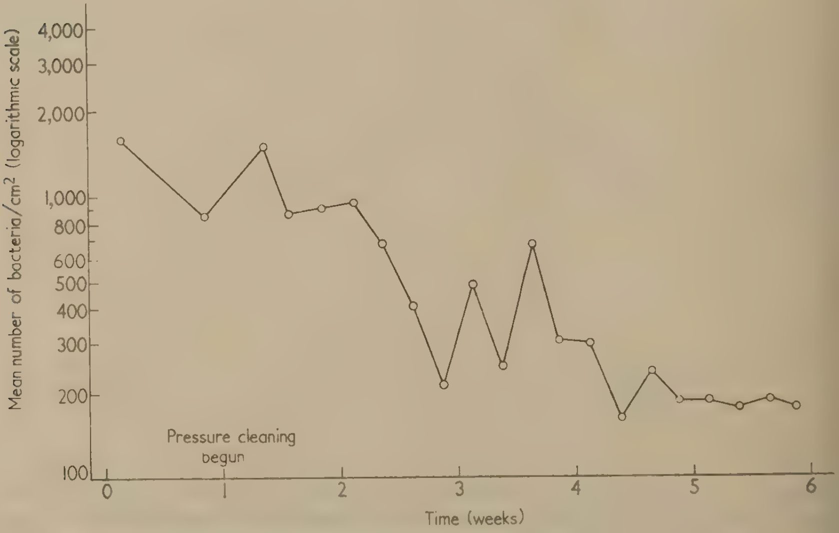

Fig. 3. The effect of the introduction of pressure cleaning on the bacterial numbers on the rind side of pig carcases.

In the fourth week of the experiment unusually large numbers of pigs were slaughtered, so that it was difficult to clean carefully enough and the counts tended to rise. Nevertheless the numbers of bacteria during that week were lower than before the use of pressure cleaners.

In the fifth week of the experiment the supply of pigs was normal again and at the same time the oilcloth aprons used in the plant were replaced by new aprons of plastic material. The numbers of bacteria during this week decreased to less than 200/cm², and in the sixth week the numbers of bacteria were even lower.

The examination shows that it was possible by more efficient cleaning of the equipment to reduce the average number of bacteria on the rind side of the dressed carcases from about 1,700/cm² to about 180/cm². This means a reduction of about 90%.

In addition to the first 10 pigs slaughtered every day the last 10 were also examined. In Fig. 4 a comparison is made between the weekly mean numbers of bacteria on those slaughtered first and on those slaughtered last, during the period when pressure cleaning was in operation.

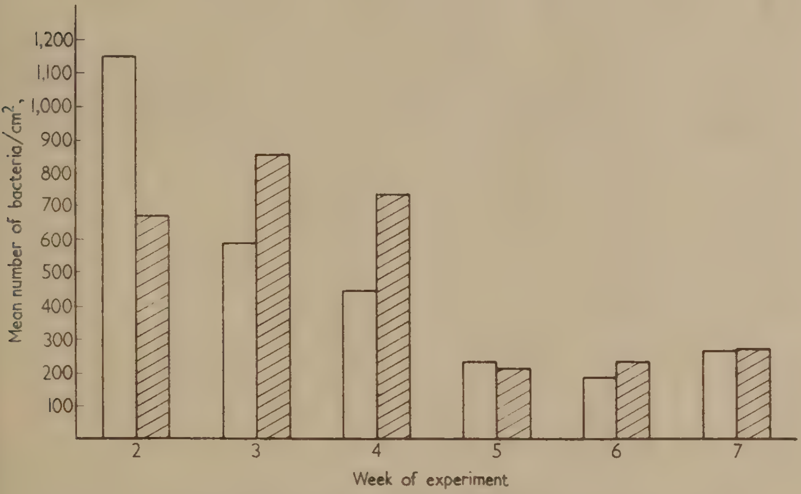

Fig. 4. A comparison between the weekly mean numbers of bacteria on 10 pigs slaughtered at the beginning of the day (plain areas) and at the end of the day (hatched areas), during a period of six weeks of pressure cleaning in a seven week experiment.

In the second week of the experiment, when the pressure cleaner had just been brought into use, the number of bacteria on the pigs slaughtered first was considerably higher than that on those slaughtered last. This is characteristic of a plant insufficiently cleaned. When the equipment is insufficiently cleaned, the pigs killed first become heavily contaminated. In the course of the day the bacteria on the equipment and machinery are removed by water etc., but some of them are transferred to the carcases. Thus, machinery and equipment become gradually cleaner in the course of the day and consequently the last pigs are cleaner than those killed first.

In the third and fourth weeks the picture was reversed, as the pigs killed first were cleaner than those killed last. The reason for this is that because all the equipment and machinery were cleaned at the end of the working day with the pressure cleaner, the pigs killed first the following morning became only very slightly contaminated. However, there were other sources of contamination which increased during the working hours and affected the pigs slaughtered later in the day. Also, in the fourth week abnormally large numbers of pigs were handled.

In the three following weeks, when the supplies of pigs were normal, the pressure cleaner was functioning satisfactorily and new plastic aprons were in use, the numbers of bacteria both on the pigs slaughtered first and on those killed last were brought to approximately the same very low level.

In the sixth and seventh weeks an attempt was made to see whether treating the whole plant with pressure cleaners during the lunch hour would effect a further improvement, but nothing appeared to be gained by this extra cleaning.

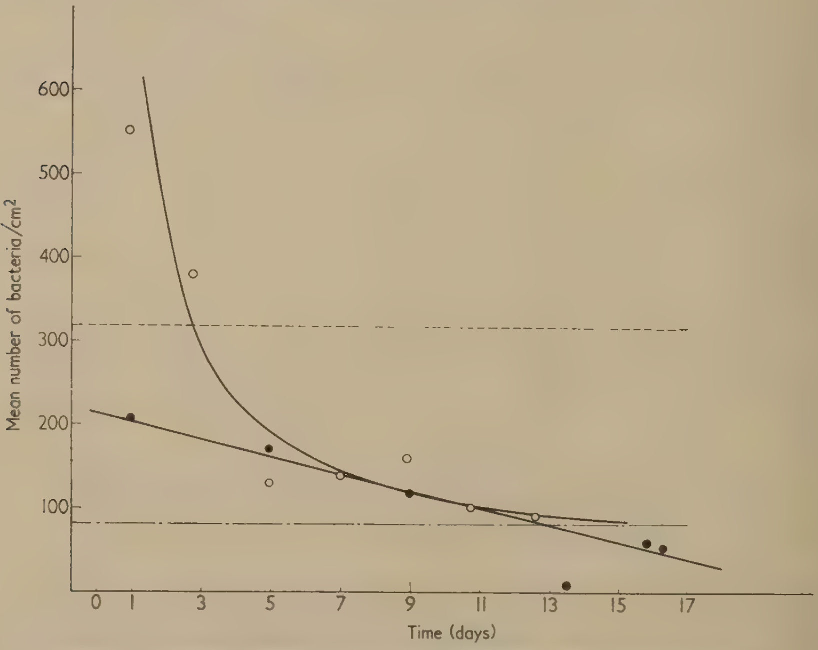

Fig. 5. The effect of pressure cleaning on the bacterial numbers on boxes and cutting tables.
Normal cleaning: boxes, dashes; cutting tables, chain dots. Pressure cleaning: boxes, open circles; cutting tables, full circles.

Cleaning of wooden boxes and cutting tables. As may be seen from Fig. 5, the pressure cleaning resulted at first in bacterial counts which were higher than after the normal cleaning procedure. This was no doubt due to the fact that to a certain extent the pressure cleaner washes out old dirt from cracks and corners which are normally difficult to clean. After a few cleanings the numbers of bacteria became considerably smaller than after normal cleaning.

General remarks. Cleaning with pressure cleaners, besides giving lower numbers of bacteria after a short time, was quicker and more easily done than normal cleaning. The saving in time will make it possible to do a thorough cleaning even on the busiest days. At the same time cleaning with pressure cleaners is more gentle to the equipment, corrosion problems are reduced and wooden equipment is less worn.

The author wishes to thank the Sellers Injector Corp., Philadelphia, U.S.A. and their Danish agents, Messrs. Tofte & Jørgensen, Copenhagen, for delivering and installing the pressure cleaners used in the experiment. For invaluable technical assistance the author is indebted to Mr. Mintaus Kilevics and Mrs. Bodil Carstensen.

REFERENCES

BLISS, I. C. (1937). The calculation of the time-mortality curve. *Ann. appl. Biol.* **24**, 815.

SILLIKER, J. H., ANDREWS, H. P. & MURPHY, J. F. (1957). A new non-destructive method for the bacteriological sampling of meats. *Food Tech., Champaign*, **11**, 317.

EMPEY, W. A. & SCOTT, W. J. (1939). Investigations on chilled beef. I. Microbial contamination acquired in the meatworks. *Bull. Coun. sci. industr. Res. Aust.* no. 126.

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TAYLOR, J. (1962). *J. appl. Bact.* 25 (1), 54–61.

THE ESTIMATION OF NUMBERS OF BACTERIA BY TENFOLD DILUTION SERIES

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SUMMARY. Results obtained by dilution series are sometimes so unlikely that doubt is cast on the validity of the method. Criteria for the rejection of results are discussed and a Table is given of acceptable results for tests with five tubes for each of three tenfold dilutions. Methods of estimating the concentration of bacteria for other numbers of tubes or of dilutions are suggested. The inherent lack of precision of the dilution series method is stressed.

THE NUMBER of viable bacteria in a suspension may be estimated by several methods of which the plate colony count is the most precise, but this cannot always be used. No suitable medium may be available, or plates having a convenient number of colonies for counting may also contain solid particles of the sample which cannot be distinguished with certainty from bacteria, or the number/ml may be too low for counting on a plate. It is then desirable to adopt the dilution series method. Certain dilutions of the suspension are prepared, and measured samples of each of these are placed with a medium in several tubes. After incubation the numbers of positives (i.e. tubes showing growth or a given reaction) are recorded. Two assumptions must be made: that the bacteria were randomly distributed in the suspension, without clumping, and that the presence of one or more bacteria in a tube always gives a positive reaction. Thus the suspension must be mixed thoroughly, and the media and conditions must be suitable and uniform. The usual estimate of the concentration of bacteria is called the most probable number (MPN) and is obtained by the statistical method of maximum likelihood. The method was described by Halvorson & Ziegler (1933a, b) and Finney (1952) has given it in a form suitable for routine calculation.

Attention has been concentrated on series with tenfold dilutions, where only three successive dilutions contribute an appreciable amount of information. For the case involving five tubes for each dilution, McCrady (1918) gave Tables of the MPN for many of the possible results. Hoskins (1933, 1934) extended this Table to cover all possible results; his Table is quoted by Prescott, Winslow & McCrady (1946) and by the American Public Health Association (Methods, 1946). Norman & Kempe (1960) produced Tables for eight tubes/dilution and outlined a computer programme which can be used for other cases. Halvorson & Ziegler (1933b) drew up a Table for some results with ten tubes/dilution. Fisher & Yates (1957) gave a Table from which an estimate having 88% of the efficiency of the MPN can be made from sufficiently long series with twofold, fourfold, or tenfold dilutions.

This paper deals with three problems encountered with tenfold dilutions. These are first, the rejection of unusual results; second, rapid estimation for other than five, eight, or ten tubes/dilution; and third, rapid estimation from more than three dilutions.

ANALYSIS OF PROBLEMS

The rejection of unusual results

Some results are so improbable that they must cast doubt on the assumption that bacteria are distributed randomly and that the presence of one or more bacteria in a tube always results in growth. For example, it is virtually impossible for there to be no positives at the highest of three concentrations in a tenfold dilution series and for all tubes to be positive at the lowest concentration. Hoskins' (1934) Tables give an MPN for such results, but it would be better to conclude that the assumptions do not hold and that no estimate can be made. A rule for the rejection of improbable results is required.

Existing tests. The χ^2 test can be used in large experiments, but it is inaccurate for the numbers of tubes which are used in practice. Stevens (1958) and Moran (1958) suggested a test based on the number of steps between the highest concentration at which some tubes show no growth and the lowest concentration with any positives. Norman & Kempe (1960) included in their Tables all results which have a probability of at least 0·00005, if the true concentration is the same as the MPN calculated from those results. Moran (1954*a*,*b*) proposed a test based on $\Sigma f(n-f)$, where there are n tubes for each dilution and f of them are negative.

The Stevens-Moran test is useful if several dilutions have been tested and if a breakdown of assumptions causes positives to occur at a concentration well beneath that at which positives would be expected, or causes negatives at a concentration for which all tubes would be expected to be positive. It is unsatisfactory for tenfold dilutions, and cannot be applied if only three dilutions are tested. The test used by Norman & Kempe is reasonable in principle, though the probability level seems too low. The $\Sigma f(n-f)$ test is relevant to assays of viruses, in which the chance of infection may vary between virus particles or between hosts. Armitage & Spicer (1956) showed that whereas this test was not very sensitive, no simple alternative was better. The test is unsuitable for the detection of irregular deviations from the normal pattern of results, such as that mentioned at the start of this section.

General principles for rejection tests. If dilution series are used for routine determinations in a laboratory, a test should be judged by the proportion (P) of determinations for which the results are wrongly rejected when the assumptions are in fact satisfied, and by the proportion of determinations for which they are correctly rejected when the assumptions are not satisfied. We should like the significance level P to be the same for any concentration of bacteria in the suspension. This is not possible, so the upper limit of P over all concentrations must be used. A test has been devised, and P has been determined, for the case involving five tubes/dilution.

Test criterion. For any concentration (C) the probability of each possible result can be determined. We can select the least probable results and continue selecting them, in order of increasing probability, until the sum of their probabilities reaches some prescribed value P' (e.g. 0·05). The remaining results, which have greater probabilities (totalling $1-P'$), will be considered *acceptable* for concentration C. Some of the improbable results are such as to cast doubt on the assumptions, but others are improbable only in relation to the concentration C. Thus a result of 0, 0, 5 would always be improbable, but 5, 5, 1 would be improbable for low values of C but reasonable for some higher values. We therefore repeat the process for all concentrations and obtain a list of results which are acceptable for at least one concentration. It is now proposed that we should reject all results which are not in this list. Since some of the results which are unacceptable for C will be acceptable for other concentrations, the probability of obtaining a result on the acceptable list will be rather greater than $(1-P')$, for any concentration.

Results. The convention is followed that we estimate the concentration in 100 ml of the suspension, by placing in tubes the equivalents of 10 ml, 1 ml and 0·1 ml of suspension. For concentrations of 8, 16, 32, 64, 128, 256 and 512/100 ml, the probability of each possible result was calculated and lists of acceptable results were prepared for values of P' equal to 0·05 and 0·10. A few additional calculations were required for results which might be acceptable at concentrations not included in the above list. Table 1 gives the acceptable results, and also gives the corresponding MPNs (taken from Hoskins' tables) and their logarithms.

Table 1. *A list of acceptable results with the corresponding MPNs and their logarithms for a series of three tenfold dilutions*

No. of tubes positive out of 5 for sample volumes (ml) of			MPN (/100 ml)	Log$_{10}$ (MPN)	No. of tubes positive out of 5 for sample volumes (ml) of			MPN (/100 ml)	Log$_{10}$ (MPN)
10	1	0·1			10	1	0·1		
0	0	0	0	—	5	0	1	31	1·491
0	1	0	1·8	0·255	5	1	0	33	1·519
1	0	0	2·0	0·301	5	1	1	46	1·663
1	1	0	4·0	0·602	5	2	0	49	1·690
2	0	0	4·5	0·653	5	2	1	70	1·845
2*	0	1	6·8	0·833	5*	2	2	95	1·978
2	1	0	6·8	0·833	5	3	0	79	1·898
2*	2	0	9·3	0·968	5	3	1	110	2·041
3	0	0	7·8	0·892	5*	3	2	140	2·146
3*	0	1	11	1·041	5	4	0	130	2·114
3	1	0	11	1·041	5	4	1	170	2·230
3	2	0	14	1·146	5	4	2	220	2·342
4	0	0	13	1·114	5*	4	3	280	2·447
4	0	1	17	1·230	5	5	0	240	2·380
4	1	0	17	1·230	5	5	1	350	2·544
4	1	1	21	1·322	5	5	2	540	2·732
4	2	0	22	1·342	5	5	3	920	2·964
4*	2	1	26	1·415	5	5	4	1600	3·204
4	3	0	27	1·431	5	5	5	>1800	—
5	0	0	23	1·362					

* These results are not acceptable at $P'=0{\cdot}10$; all results in the table are acceptable at $P'=0{\cdot}05$.

Actual significance levels. Table 2 gives the probability of obtaining a result in the above list, for various concentrations. As explained earlier, these probabilities are greater than $(1-P')$.

Table 2. *The probability of obtaining results on the acceptable list given in Table* 1

Concn of bacteria (/100 ml)	Probability for P' values of	
	0·05	0·10
8	0·978	0·949
16	0·978	0·958
32	0·984	0·974
64	0·989	0·969
128	0·991	0·947
256	0·993	0·961
512	0·997	0·990

The probability depends on the concentration of bacteria, the minimum values being 0·978 and 0·947. Thus the significance level of the test (i.e. the maximum value of P, the proportion of incorrect rejections when the assumptions are satisfied) is 0·022 if $P'=0{\cdot}05$ and 0·053 if $P'=0{\cdot}10$. By varying P' it would be possible to obtain any desired significance level, but these are sufficiently near to the conventional levels for practical purposes.

Comparison with the test of Norman & Kempe (1960). If all results are accepted which have a probability of at least 0·00005 at their MPN, the list is much longer and the significance level is very small. The latter is certainly less than 0·005, and the test will be inefficient since it will not reject many results when the assumptions are not satisfied. If the probability were fixed at a higher level, the significance level would be greater. For the case considered above, a probability of 0·013 would give almost the same list of acceptable results as Table 1 ($P'=0{\cdot}10$), and 0·007 would give almost the same list as Table 1 ($P'=0{\cdot}05$). It is easier to calculate the probability for the MPN than to apply the test used in this paper. It is suggested that in future calculations of Tables with no more than 15 tubes/dilution any result should be accepted which has a probability greater than 0·005 for the MPN. This should give a significance level of the order of 0·01–0·05. If there are more than 15 tubes/dilution the χ^2 test is probably preferable.

Tests involving three dilutions and more than five tubes/dilution

Tables are available for 8 or 10 tubes for each dilution. They could be prepared for other numbers, but this will not be worth doing for an occasional experiment. Finney's method (Finney, 1952) can be used to obtain the exact MPN, but a close approximation can generally be obtained quickly by reducing the scale of the results and using Table 1. For each dilution we calculate the proportionate number of positive tubes out of 5. If this gives whole numbers we can read the MPN directly from Table 1; otherwise we use linear interpolation in the logarithms of MPNs.

Example:

Volume (ml)	No. of tubes positive out of 15	Proportionate no. of tubes positive out of 5
10	13	$4\frac{1}{3}$
1	2	$\frac{2}{3}$
0·1	0	0

The values of $\log_{10}$ (MPN) with no positives in the 0·1ml dilutions thus become:

No. of 1 ml tubes positive	Calculated $\log_{10}$ (MPN) for indicated nos. of 10 ml tubes positive		
	4	$4\frac{1}{2}$	5
0	1·114	1·197	1·362
$\frac{2}{3}$		1·283	
1	1·230	1·326	1·519

The underlined figures are taken from Table 1, and the other figures are obtained by interpolation. The interpolated $\log_{10}$ (MPN) is 1·283, corresponding to an MPN/100 ml of 19·2. The exact MPN, calculated by Finney's method, is 18·4.

Rejection of results. If all the results required for interpolation do not have an asterisk in Table 1, there is little doubt that the results are acceptable. In other cases the MPNs needed for interpolation can be found in the Tables of Hoskins (1934), the interpolated MPN calculated, and the probability of the results at the MPN obtained. If this is less than 0·005, the results should be rejected.

Comparison with exact values. The method has been applied to nine sets of experimental data which satisfy the conditions of the last paragraph, and the results are given in Table 3. The exact MPN for each set was calculated by the method given by Finney (1952).

Table 3. *A comparison of exact results (a) with those given by the interpolation method (b)*

Series no.	No. of tubes* positive for sample volumes (ml) of			$\log_{10}$ (MPN)		MPN (/100 ml)	
	10	1	0·1	(a)	(b)	(a)	(b)
1	13	2	0	1·265	1·283	18·4	19·2
2	15	7	2	1·862	1·860	73	72
3	15	5	1	1·681	1·683	48	48
4	15	9	3	2·036	2·041	109	110
5	13	4	0	1·342	1·370	22	23
6	15	6	3	1·845	1·845	70	70
7	15	15	5	2·686	2·669	485	467
8	15	11	5	2·239	2·240	173	174
9	15	8	4	2·015	2·014	104	103

* Tests involving 15 tubes/dilution.

The greatest difference between the exact and interpolated logarithms is 0·028, which is negligible when compared with the inherent variations in estimates from dilution series.

Tests involving more than three dilutions

Tests involving only three successive dilutions give useful information, and the most concentrated of these should contain between 1·6 and 16 organisms/tube. But unless we have a fairly good preliminary estimate it is necessary to prepare more than three dilutions. For 5 tubes/dilution, Prescott *et al.* (1945) suggested a way of making rapid estimates by using the Tables for only three dilutions. The following rules are suggested as an alternative and they will often give the same estimate, but provide a more critical approach in difficult cases.

First, if some dilutions give all tubes positive, include in the calculations only the most dilute of these.

Example:

Volume (ml)	No. of tubes positive out of 5	
10	5	
1	5	
0·1	5	Calculate from these dilutions
0·01	1	
0·001	0	

Second, if some dilutions give no tubes positive, include in the calculations only the most concentrated of these.

Example:

Volume (ml)	No. of tubes positive out of 5	
10	5	Calculate from these dilutions
1	3	
0·1	0	
0·01	0	
0·001	0	

Third, if application of the first two rules mentioned above leaves three dilutions, use Table 1 to estimate the concentration; if such results are not in Table 1, reject the data.

Example:

Volume (ml)	No. of tubes positive out of 5	
10	5	
1	5	Calculate from these dilutions
0·1	2	
0·01	0	
0·001	0	

Fourth, if application of the first two rules leaves four dilutions, take both sets of three consecutive dilutions from them, find the logarithm of the MPN from Table 1, and take the average of these logarithms as the final $\log_{10}$ (MPN). If either set of three dilutions is not in Table 1, the MPN can be found by using Hoskins' Tables. In such a case, or if the logarithms to be averaged differ by more than 0·1, the probability of the results at the MPN should be calculated and if less than 0·005 the results should be rejected.

Example:

Volume (ml)	No. of tubes positive out of 5
10	5
1	5
0·1	4
0·01	3
0·001	0

By the first rule, 10 ml may be excluded. From Table 1, a result of 5, 4, 3 gives 2·447 as the log of the number in 10 ml and 4, 3, 0 gives 1·431 as the log for 1 ml and hence 2·431 for 10 ml. The average is thus 2·439, corresponding to an estimated

MPN of 275/10 ml or 2,750/100 ml. The exact MPN, calculated from all 5 dilutions, is 2,710/100 ml.

In this particular case, the results 5, 4, 3 are asterisked in Table 1. The probability of the results (for all 5 dilutions), if the concentration is 2,750/100 ml, is 0·017. This is greater than 0·005, so the results can be accepted.

Fifth, if the first two rules do not exclude any dilutions, the results should be rejected (Stevens' test is then significant, at the $P=0{\cdot}05$ level).

Example:

Volume (ml)	No. of tubes positive out of 5
10	5
1	4
0·1	2
0·01	1
0·001	0

These results should be rejected.

Comparison with exact values. The method has been applied to thirteen sets of experimental data which satisfied the criteria for acceptability. The exact MPN for each set was calculated by the method given by Finney (1952). The results are given in Table 4.

The greatest difference between exact and estimated logarithms is 0·044 (series 11). It may be noted that this series might have been rejected by a more exact test of significance, since the probability of the results at the MPN was only 0·0064. The next greatest difference was only 0·023.

Table 4. *A comparison of exact results* (a) *with those obtained by estimation* (*b*)

Series no.	No. of tubes* positive for sample volumes (ml) of					Log_{10} (MPN)		MPN (/100 ml)	
	10	1	0·1	0·01	0·001	(a)	(b)	(a)	(b)
1	5	5	0	0	0	2·364	2·362	231	230
2	5	4	1	0	0	2·228	2·230	169	170
3	5	5	2	1	0	2·841	2·839	693	690
4	5	5	2	0	0	2·689	2·690	489	490
5	5	5	3	1	0	3·037	3·041	1,090	1,100
6	5	5	1	0	0	2·515	2·519	327	330
7	5	5	5	1	0	3·517	3·519	3,290	3,300
8	5	5	5	2	0	3·694	3·690	4,940	4,900
9	5	5	2	2	0	2·971	2·973	935	940
10	5	5	5	1	1	3·661	3·663	4,580	4,600
11	5	5	3	1	1	3·138	3·094	1,370	1,240
12	5	5	4	3	0	3·433	3·439	2,710	2,750
13	5	5	1	1	0	2·655	2·632	452	429

* Tests involving 5 tubes/dilution.

Other numbers of tubes. If we have more than three dilutions and more than 5 tubes/dilution, the MPN can be estimated by a combination of the methods in this section and in the last one.

DISCUSSION

The fact that we can make rapid estimates of bacterial numbers by using dilution series must not blind us to the inherent lack of precision of the method. Cochran (1950) gave a Table which showed that for tenfold dilutions with five tubes/dilution the standard error of the $\log_{10}$ (MPN) is approximately 0·259 and that confidence limits on the concentration are approximately 0·30 times and 3·30 times the MPN. The investigation on which Table 1 is based showed that the standard error of $\log_{10}$ (MPN) lies between 0·22 and 0·25, except at very low concentrations, and that confidence limits are about 0·26 and 2·60 times the MPN. These values are close to Cochran's, and confirm that the estimate is very imprecise. It can be improved by using a smaller dilution ratio, or by using more tubes at each dilution; but for a given expenditure of effort it will always be less precise than a colony count, if the latter is possible.

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REFERENCES

ARMITAGE, P. & SPICER, C. C. (1956). The detection of variation in host susceptibility in dilution counting experiments. *J. Hyg., Camb.*, **54**, 401.

COCHRAN, W. G. (1950). Estimation of bacterial densities by means of the "most probable number". *Biometrics* **6**, 105.

FINNEY, D. J. (1952). *Statistical Method in Biological Assay*. London: C. Griffin.

FISHER, R. A. & YATES, F. (1957). *Statistical Tables for Biological, Agricultural and Medical Research*, 5th ed. Edinburgh: Oliver and Boyd.

HALVORSON, H. O. & ZIEGLER, N. R. (1933*a*). Application of statistics to problems in bacteriology. I. A means of determining bacterial population by the dilution method. *J. Bact.* **25**, 101.

HALVORSON, H. O. & ZIEGLER, N. R. (1933*b*). Application of statistics to problems of bacteriology. II. A consideration of the accuracy of dilution data obtained by using a single dilution. *J. Bact.* **26**, 559.

HOSKINS, J. K. (1933). The most probable numbers of *B. coli* in water analysis. *J. Amer. Wat. Wks Ass.* **25**, 867.

HOSKINS, J. K. (1934). Most probable numbers for evaluation of coli-aerogenes tests by fermentation tube method. *Publ. Hlth Rep., Wash.*, **49**, 393.

MCCRADY, M. H. (1918). Tables for rapid interpretation of fermentation-tube results. *Canad. publ. Hlth J.* **9**, 201.

METHODS (1948). *Standard Methods for the Examination of Dairy Products*, 9th ed. New York: American Public Health Association.

MORAN, P. A. P. (1954*a*). The dilution assay of viruses. *J. Hyg., Camb.*, **52**, 189.

MORAN, P. A. P. (1954*b*). The dilution assay of viruses. II. *J. Hyg., Camb.*, **52**, 444.

MORAN, P. A. P. (1958). Another test for heterogeneity of host resistance in dilution assays. *J. Hyg., Camb.*, **56**, 319.

NORMAN, R. L. & KEMPE, L. L. (1960). Electronic computer solution for the MPN equation used in the determination of bacterial populations. *J. biochem. microbiol. Technol. Engng* **2**, 157.

PRESCOTT, S. C., WINSLOW, C. A. & MCCRADY, M. H. (1945). *Water Bacteriology*, 6th ed. New York: John Wiley.

STEVENS, W. L. (1958). Dilution series: a statistical test of technique. *J.R. statist. Soc.* B**20**, 205.

(*Received* 31 *August*, 1961)

White, A. & White, H. R. (1962). *J. appl. Bact.* **25** (1), 62–71.

SOME ASPECTS OF THE MICROBIOLOGY OF FROZEN PEAS

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SUMMARY: The number of bacteria present in commercially frozen peas was found to be usually between 10^4 and 10^6 g. Most of the organisms were of two species, *Leuconostoc mesenteroides* and *Streptococcus lactis*. After thawing the leuconostocs multiplied more rapidly than the streptococci and in 48 hr the former constituted almost the entire population.

Growth studies of four isolates of each of the above species, both in laboratory media and on blanched peas, showed no difference in growth rates before and after freezing. When some organisms, including potential pathogens, were inoculated on to peas rapid multiplication took place even in the presence of leuconostocs or streptococci.

It is a common belief that frozen produce when thawed is more perishable than fresh produce. If this is so there are at least two possible reasons: first, that plant or animal cells which have been frozen and thawed are more suitable for bacterial growth owing to breakdown of cell structure and release of free fluid and nutrients; second, that freezing has a direct effect on the growth rate of bacteria.

The published results relating to this subject are somewhat contradictory. Kallert (1928) studied the bacteriology of minced meat and concluded that frozen meat was not more perishable than fresh meat, whereas Gressel & Gräfe (1929) who continued this work found that it was. Hartsell (1951) studied the growth of four bacterial species in frozen whole eggs and found that these bacteria multiplied more rapidly after freezing and thawing. He suggested that this was due to a physiological stimulation of bacterial cells which was brought about by freezing. Sulzbacher (1952) inoculated a psychrophilic pseudomonad on to meat before freezing it, and found that the lag phase on frozen meat after thawing was longer than on the fresh meat and also that growth was slower on thawed frozen meat.

Squires & Hartsell (1955) investigated the survival of *Escherichia coli* in different frozen storage menstrua and found that after such storage the generation times during subsequent growth varied according to the medium. Kitchell & Ingram (1956) investigated the growth of bacteria on meat, but found no evidence that the liberation of free fluid in thawed tissue favoured bacterial multiplication or that bacteria built up a 'growth potential' in the frozen state. They concluded that frozen meat was neither more nor less perishable than the corresponding fresh meat. Further work (Kitchell & Ingram, 1959) supported this conclusion.

The present work is confined to certain aspects of the bacteriology of frozen peas.

Experimental Methods

The numbers of bacteria present in commercial frozen peas. In all, 107 packets of one brand of commercial frozen peas were examined. Each sample, which consisted of approximately 1 g of peas, was macerated for 2 min in an M.S.E. macerator (Measuring and Scientific Equipment Ltd., 14-28, Spenser St., London, S.W.1) run at maximum speed, before being plated for colony counts. Several plating media were tried: glucose-Yeastrel agar, nutrient agar, tomato juice agar, malt extract agar, Rogosa's medium (Rogosa, Mitchell & Wiseman, 1951), V8 vegetable juice (Campbells Soups Ltd., Kings Lynn, Norfolk) medium and a pea extract medium. Glucose-Yeastrel agar (GYA) (peptone, 1 g; Yeastrel, 0·3 g; glucose, 1 g; NaCl, 0·25 g; agar, 1 g; distilled water, 100 ml; pH 7·6) was chosen as the most suitable general growth medium. It gave higher counts than all the other media used and was readily available in the laboratory; other authors have reported this medium to be most suitable for counting bacteria in thawed food products (Goresline, 1946; Hartsell, 1951). It was found that 30° was the most suitable temperature for incubation and this was used throughout.

The microflora of commercial frozen peas and the effect of storage at high temperatures. Statistical picks were carried out on the colonies obtained on GYA from three packets of a single brand of commercial frozen peas. The packets were sampled while frozen and forty colonies were picked by the disc method described by Harrison (1938) from the plates obtained from each sample. Each of these cultures was then identified.

The types of organisms present in forty packets of frozen peas were determined by another method. Counts were obtained using the Miles & Misra (1938) counting technique modified by the use of platinum tipped pipettes (Davis & Bell, 1958, 1959). Two media were employed; these were GYA and a medium containing sucrose (Garvie, pers. comm.) (tryptone, 1 g; Yeastrel, 0·5 g; K_2HPO_4, 0·5 g; sucrose, 5 g; ammonium citrate, 0·2 g; agar, 1 g; pH 6·8). Slime forming colonies could be counted on the sucrose medium and in the statistical picks described above it had been found that all slime formers present were leuconostocs. Colonies which did not form slime on this medium were taken to be streptococci if they were of the typical colonial morphology and size, since more detailed examination of a sample of colonies indicated this to be a fair assumption. The results obtained on GYA were used to obtain the total count.

In order to determine which organisms multiplied during the storage of peas after removal from the deep freeze and were responsible for spoilage, further statistical picks were carried out on plates prepared from single packets before and after incubation at 30° and 22° for 18 and 48 hr respectively. (Incubation for 18 hr was chosen as it was usually the time at which spoilage first became apparent at 30°; after 48 hr spoilage was much more advanced).

Growth studies with laboratory media and peas. From one of the statistical picks just described, 8 isolates were selected as representative of the species present. Four of the isolates were identified as *Leuconostoc mesenteroides* and four as *Streptococcus lactis*, although two of the streptococci failed to ferment lactose. Experiments were done on these 8 isolates to observe the effect of freezing on growth rates in laboratory media and on fresh and thawed frozen peas (both blanched and unblanched). Counts were carried out by the Miles & Misra technique modified as before. All material was frozen by placing in a deep freeze at −23°.

Growth rate studies in laboratory media were done in triplicate in the broth equivalent of GYA. The inocula used were: first, 0·01 ml of an 8 hr culture in 10 ml of broth; and second, 0·01 ml of a similar 8 hr culture which had been kept frozen for 14 days, then thawed in a water bath at 37° for a few minutes before inoculation into broth.

The peas were shelled in the laboratory under aseptic conditions. They were blanched by immersing in boiling water for 1 min and then simultaneously cooled and inoculated by transference to a litre of cold water to which the inoculum, 5 ml of an 8 hr culture, had been added. In this the peas were left for 3 min, and they thus acquired an infection of 10^3–10^4 organisms/g. The peas were then placed in sterile wax cartons. Three of these were incubated at 30° and the growth of the bacteria in the peas was followed. The remainder were frozen. One was removed from the freezer after 6, 12, 18 and 24 weeks, respectively, and taken directly to an incubator at 30° for the determination of growth rates. This process was repeated with fresh unblanched peas.

The growth of organisms other than the normal flora. It is often thought that the presence of the so-called 'normal' flora of frozen produce is a safeguard against public health hazards and that initially these organisms are likely to outnumber any pathogenic organisms which might be introduced in the factory or elsewhere and prevent their increase to a dangerous level. It was thought of interest, therefore, to investigate the ability of some organisms not normally found on the peas to multiply on thawed frozen peas, and to establish their growth rates. The multiplication of these organisms on thawed frozen peas in the presence of streptococci or leuconostocs was also investigated.

Five organisms were selected for these studies: two from the National Collection of Type Cultures—*Staphylococcus aureus* (NCTC 7447) and *E. coli* (NCTC 86); and three from the stock culture collection of this laboratory—*Salmonella typhi-murium*, *Bacillus subtilis* and *Strep. faecalis*. Each organism was inoculated on to peas which had been previously prepared, blanched and frozen in the laboratory. The inoculations were carried out as before, to give concentrations of 10^3–10^4 organisms/g.

Mixed cultures were also used as inocula. Five experiments were carried out using the following combinations of the above stock cultures and strains of leuconostoc and streptococci isolated from frozen peas: *Staph. aureus* and *Leuc. mesenteroides* (strain 101); *Salm. typhi-murium* and *Leuc. mesenteroides*; *B. subtilis* and *Leuc. mesenteroides*; *E. coli* and *Leuc. mesenteroides*; *E. coli* and *Strep. lactis* (strain 118). In each case approximately equal numbers of the two organisms were inoculated and the development of both organisms was followed for 20 hr.

RESULTS

The microflora of commercial frozen peas and the effect of storage at high temperatures

The numbers and types of bacteria present. The bacterial counts on packets of frozen peas varied from 10^3 to 10^7/g. Out of the 107 packets examined, 58 had counts between 10^4 and 10^5/g and a further 23 had counts between 10^5 and 10^6/g.

The statistical picks of the colonies obtained by plating frozen peas showed that the main types present were catalase negative Gram-positive cocci. The commonly found organisms were studied in more detail. They fell into two groups, one of which consisted of heterofermentative, catalase negative, Gram-positive cocci which had no action on litmus milk and generally produced slime on sucrose media; these organisms were provisionally identified as leuconostocs. The strains included in the second group were homofermentative, catalase negative, Gram-positive cocci, which reduced litmus, produced ammonia from arginine and did not produce slime on sucrose media. These were identified as streptococci.

In 27 of the 40 packets examined leuconostocs constituted between 40% and 60% of the population; in the remaining 13 packets the leuconostocs formed as few as 10% or as many as 90% of the total organisms. The rest of the population consisted of streptococci, except for a small percentage of other organisms; this was usually less than 10% of the total population and consisted of Gram-negative rods and some sporeformers.

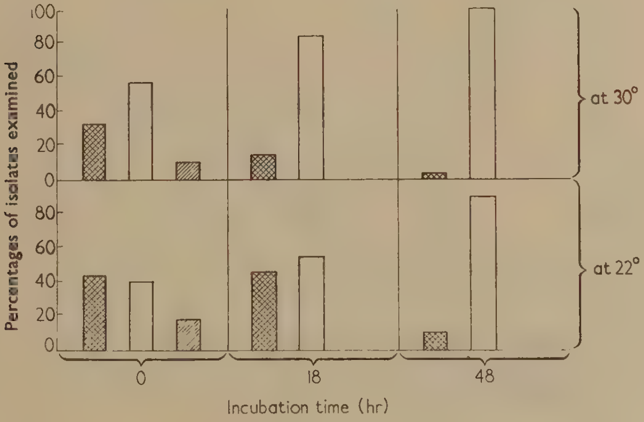

Fig. 1. **The development of the flora of commercial frozen peas on incubation at two temperatures. Cross-hatched areas, streptococci; white areas, leuconostocs; hatched areas, other organisms.**

The effect of storage temperature after removal from the deep freeze. The results of the statistical studies are shown in Fig. 1. The packet of frozen peas which was incubated at 30° initially contained approximately 10^4 organisms/g, comprising 33% of streptococci and 57% of leuconostocs. After 18 hr the total count was approximately 10^8/g, and the proportions then were 15% of streptococci and 85% of leuconostocs. After 48 hr the total count was still about 10^8/g, but 97% of these were now leuconostocs.

The second packet, which was incubated at 22°, gave similar results, although initially 43% of streptococci and 40% of leuconostocs were present; after 48 hr only 10% were streptococci, the remaining 90% being leuconostocs.

Accompanying the multiplication of streptococci and leuconostocs there was always a drop in pH from about 7 to about 4. The colour change from green to yellow normally associated with spoilage of frozen peas occurred at pH 4·6.

Growth studies with laboratory media and peas

Growth in broth. The results obtained in broth are shown in Figs. 2 and 3. The results shown for *Leuc. mesenteroides* and *Strep. lactis* are typical. A variance analysis carried out on the logarithmic phase of the graphs obtained for seven isolates showed no significant difference between the gradients at the 95% level. Results were not obtained for the eighth isolate. A longer lag (2–9 hr) was shown by the cultures which were frozen and thawed before subculturing compared with those which were not frozen.

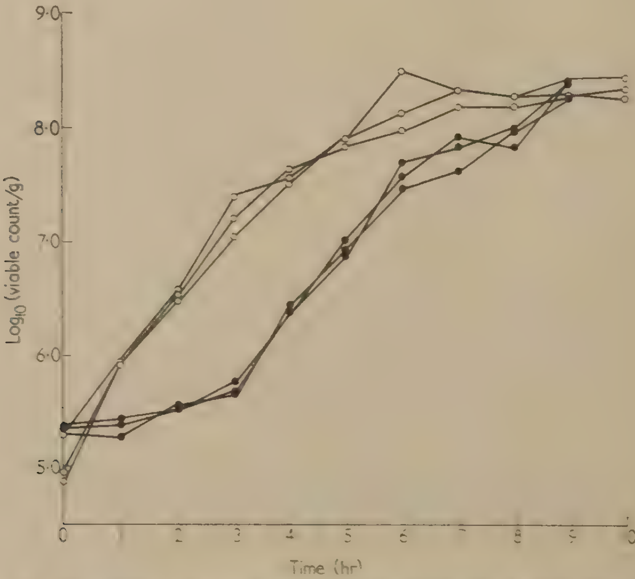

Fig. 2. The development of *Leuc. mesenteroides* (strain 101) in broth with prior freezing (full circles) and without prior freezing (open circles). Triplicate tests were done on each occasion.

Growth on peas. Figs. 4 and 5 show the results obtained for the same two cultures grown on blanched peas. These are typical of the results obtained for eight isolates. A variance analysis was carried out on the gradients of each set of graphs for eight isolates and there was no significant difference between gradients at the 95% level.

An apparent lag occurred due to the time taken for the peas to thaw before bacterial multiplication could begin. A period of approximately 2 hr was required for peas to reach 30° after they were placed in the incubator; thus with an apparent lag of $2\frac{1}{2}$ hr, the true lag would probably be less than 1 hr. The apparent increase in lag shown in Figs. 4 and 5 is between 2 and 3 hr. This increase is very much less than that which occurred when freezing took place in broth.

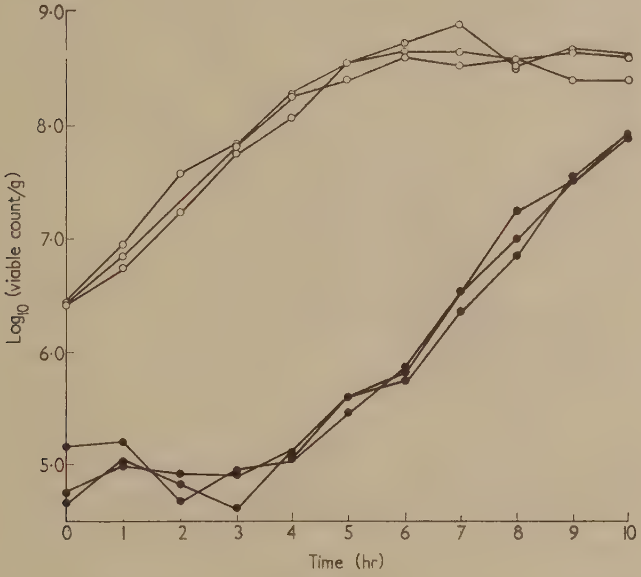

Fig. 3. The development of *Strep. lactis* (strain 118) in broth with prior freezing (full circles) and without prior freezing (open circles). Triplicate tests were done on each occasion.

The growth rate determinations carried out on unblanched peas, both fresh and frozen, indicated that no change in growth rates had been brought about by freezing, but the curves were too irregular for any calculations to be made.

The growth on peas of organisms other than the normal flora. Each of the five organisms studied grew well as pure cultures inoculated on to frozen peas, each reaching a population of approximately 10^8/g after incubation for 14–16 hr.

The results obtained when mixed cultures were inoculated on to peas showed that *E. coli*, *Staph. aureus*, *Salm. typhi-murium* and *B. subtilis* were all capable of multiplying on peas and reaching populations of about 10^8/g after incubation for 14–16 hr even in the presence of *Leuc. mesenteroides*.

A similar result was obtained for *E. coli* when inoculated on to peas with *Strep. lactis*.

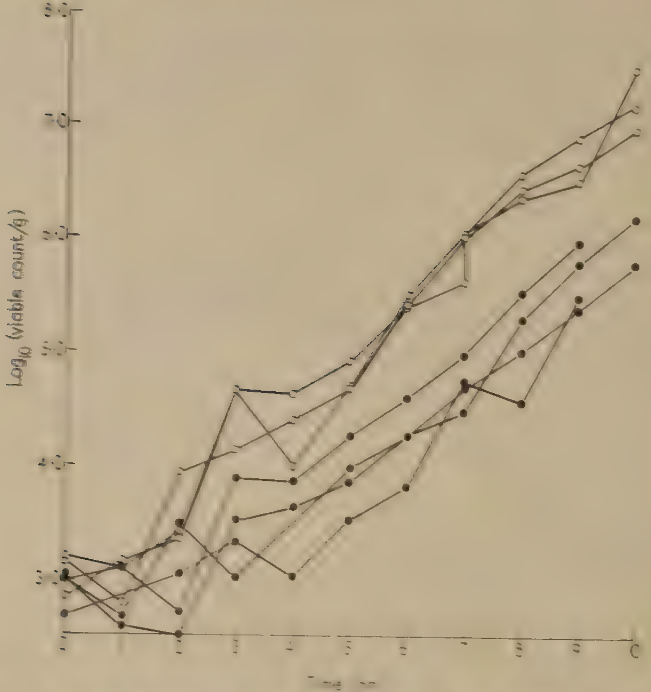

Fig. 4. The development of *Leuc. mesenteroides* (strain 191) on peas with prior freezing (full circles) and without prior freezing (open circles). Triplicate tests were done before freezing and single tests at 6-weekly intervals after freezing.

DISCUSSION

The viable counts of frozen peas which we obtained on glucose-Yeastrel agar are of the same order as those reported by Pederson (1947), who found that counts of 10^4–10^5/g on tryptone-yeast extract-glucose agar were to be expected in good quality frozen vegetables. Pederson suggested that counts of 10^6/g or more were indicative of poor handling, and it is of interest to note that very few of our counts reached this level. Many of the bacterial counts reported elsewhere in the literature are not strictly comparable with our figures since the authors either gave no indication of the culture medium used or used a less rich medium on which fewer organisms would be able to grow. For example Tressler (1938) stated that counts of below 80,000/g in frozen vegetables were satisfactory; but he did not specify the medium used.

Our evidence suggests that the dominant flora of frozen peas consists of *Strep. lactis* and *Leuc. mesenteroides* and that the spoilage of thawed frozen peas results from their multiplication. This is partly supported by the literature. Several workers (Lochhead & Jones 1938, Perry *et al.* 1948) and Hucker (1954) have recorded the presence of Gram-positive cocci on frozen vegetables but have not identified them further. However, Sanderson (1941) and Fitzgerald (1947) reported that *Strep. faecalis* occurred in frozen vegetables and attributed spoilage to the acid produced by this organism. Fitzgerald had also reported the isolation of similar strains of *Strep. faecalis* from the seed coats of fresh peas. Stark & Sherman (1935) found *Strep. lactis* on fresh peas. Only one reference has been found to the presence of leuconostocs on frozen vegetables (Borgstrom, 1955) and no references at all to their association with spoilage of these foods. This is surprising as the principal sugar present in peas is sucrose and, on incubation of commercial frozen peas, the production of slime by the leuconostocs becomes obvious after 24–36 hr.

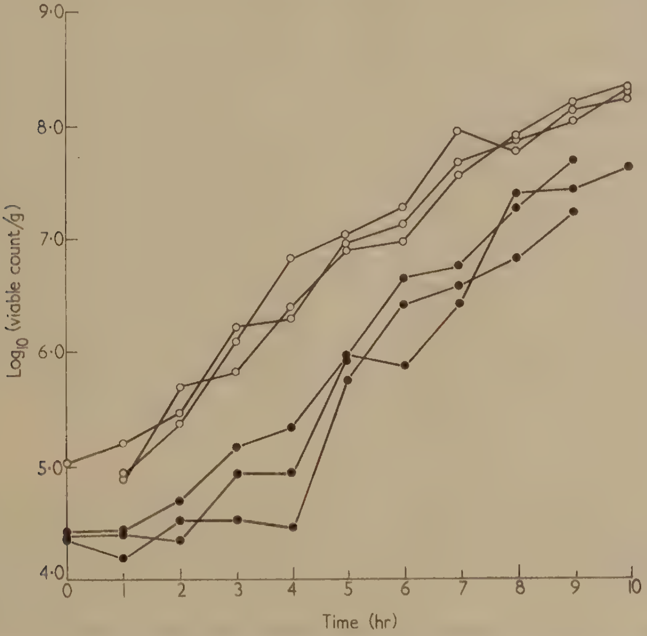

Fig. 5. The development of *Strep. lactis* (strain 118) on peas with prior freezing (full circles) and without prior freezing (open circles). Triplicate tests were done before freezing and single tests at 6-weekly intervals after freezing.

Our studies indicate that there is no change in the growth rate of cultures of *Strep. lactis* and *Leuc. mesenteroides* after freezing and thawing either in broth or on peas. However, the lag phase of the thawed frozen culture is prolonged to a considerable extent in cultures frozen in broth and slightly so in cultures frozen on peas. Thus freezing, particularly in broth culture, appears to exert a physiological effect on leuconostoc and streptococcus cells, affecting their ability to initiate growth on thawing but not affecting their subsequent rate of growth. Observations reported in the literature concerning the effect of freezing on growth rates and on length of lag phase seem to be conflicting, although it should be borne in mind that different strains, and usually different genera of bacteria, have been used by the various authors. Gram-positive cocci are noticeably more resistant to freezing than other organisms (Jones & Lochhead, 1939) and for this reason might possibly be expected to be less affected by freezing and thawing. However, among the Gram-negative organisms consistent behaviour has not been recorded. Hartsell (1951) showed that the growth of *E. coli* was stimulated in thawed frozen eggs. Squires & Hartsell (1955) found that the growth rates of *E. coli* cultures were altered after freezing and thawing in yeast extract-veal infusion broth and that the generation times changed with the length of time of freezing storage, and according to Sulzbacher (1952) the growth rate of a pseudomonad was slower and the lag period prolonged after freezing and thawing on meat. However, Kitchell & Ingram (1956, 1959), working with a pseudomonad frozen in laboratory media and in meat, obtained results similar to ours, finding no change in the generation time but an increase in length of lag after freezing. It seems that organisms in food are partially protected from the effects of freezing. This was shown in our experiments where the lag phase was extended more markedly after freezing the organisms in broth than after freezing them in peas. A similar observation was made by Kitchell & Ingram (1959), who demonstrated that when unheated aqueous extracts of meat were added to liquid laboratory media pseudomonad cells were protected, the effects of freezing being reduced.

Our results with mixed cultures, in which we showed that *E. coli*, *Staph. aureus*, *Salm. typhi-murium* and *B. subtilis* were all capable of multiplying in the presence of the normal flora of frozen peas, demonstrate the importance of factory hygiene in the production of frozen vegetables. The multiplication of the normal flora cannot be relied upon to prevent the growth of potential pathogens, at least if the latter are initially present in high numbers.

The authors wish to express their thanks to Birds Eye Foods Ltd. for supplying samples of frozen peas, and to Dr. P. M. Scholes of this Department for her valuable suggestions throughout the work and especially for her help in preparing the manuscript. They are also grateful to Miss P. Reed for her excellent technical assistance.

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REFERENCES

BORGSTROM, G. (1955). Microbiological problems of frozen food products. *Advanc. Food Res.* 6, 163.

DAVIS, J. G. & BELL, J. S. (1958). The advantages of a platinum tube dropping pipette for making colony counts. *J. appl. Bact.* 21, 217.

DAVIS, J. G. & BELL, J. S. (1959). The drop technique for colony counts in microbiology. *Lab. Pract.* 8, 38.

FITZGERALD, G. A. (1947). Are frozen foods a public health problem? *Amer. J. publ. Hlth* 37, 695.

Goresline, H. E. (1946). Microbiological examination of foods. Tentative methods for the microbiological examination of frozen foods. *Amer. J. publ. Hlth* **36**, 332.

Gressel & Gräfe (1929). Zur Frage der Haltbarkeit von Hackfleish aus Frischfleisch und Gefrierfleisch. *Berl. tierärztl. Wschr.* **45**, 430.

Harrison, J. (1938). Numbers and types of bacteria in cheese. *Proc. Soc. agric. Bact.* p. 12.

Hartsell, S. E. (1951). The growth initiation of bacteria in defrosted eggs. *Food Res.* **16**, 97.

Hucker, G. J. (1954). Low temperature organisms in frozen vegetables. *Food Tech., Champaign*, **8**, 79.

Jones, A. H. & Lochhead, A. G. (1939). A study of micrococci surviving in frozen pack vegetables and their enterotoxic properties. *Food Res.* **4**, 203.

Kallert, E. (1928). Vergleichende Untersuchungen über die Haltbarkeit von Hackfleisch aus Frischfleisch und Gefrierfleisch. *Berl. tierärztl. Wschr.* **44**, 547.

Kitchell, A. G. & Ingram, M. (1956). A comparison of bacterial growth on fresh meat and on frozen meat after thawing. *Ann. Inst. Pasteur* **8**, 121.

Kitchell, A. G. & Ingram, M. (1959). The survival of bacteria in frozen meat and their growth after thawing. *Int. Congr. Refrig., Copenhagen*, **3**, 65.

Lochhead, A. G. & Jones, A. H. (1938). Types of bacteria surviving in frozen-pack vegetables. *Food Res.* **3**, 299.

Miles, A. A. & Misra, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.*, **38**, 732.

Pederson, C. S. (1947). Significance of bacteria in frozen vegetables. *Food Res.* **12**, 429.

Perry, H., Townsend, C. T., Andersen, A. A. & Berry, J. A. (1948). Studies on *Clostridium botulinum* in frozen pack vegetables. *Food Tech., Champaign*, **2**, 180.

Rogosa, M., Mitchell, J. & Wiseman, R. F. (1951). A selective medium for the isolation and enumeration of oral and fecal lactobacilli. *J. Bact.* **62**, 132.

Sanderson, N. H. (1941). The bacteriology and sanitation of quick frozen foods. *Refrig. Engng* **42**, 228.

Squires, R. W. & Hartsell, S. E. (1955). Survival and growth initiation of defrosted *Escherichia coli* as affected by frozen storage menstrua. *Appl. Microbiol.* **3**, 40.

Stark, P. & Sherman, J. M. (1935). Concerning the habitat of *Streptococcus lactis*. *J. Bact.* **30**, 639.

Sulzbacher, W. L. (1952). Effect of freezing and thawing on the growth rate of bacteria in ground meat. *Food Tech., Champaign*, **6**, 341.

Tressler, D. K. (1938). Bacteria, enzymes and vitamins—indices of quality in frozen vegetables. *Refrig. Engng* **36**, 319.

(*Received* 23 *August*, 1961)

HUGO, W. B. & BEVERIDGE, E. G. (1962). *J. appl. Bact.* **25** (1), 72–82.

A QUANTITATIVE AND QUALITATIVE STUDY OF THE LIPOLYTIC ACTIVITY OF SINGLE STRAINS OF SEVEN BACTERIAL SPECIES

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SUMMARY: Single strains of *Serratia marcescens*, *Staphylococcus aureus*, *Micrococcus freudenreichii*, *Pseudomonas hydrophila*, *Achromobacter fischeri*, *Bacillus subtilis* and *Bacillus cereus* were examined for their lipolytic activity, qualitatively using different lipolysis detecting media and quantitatively by means of acid production. The substrates investigated were olive oil, tributyrin, ethyl laurate and ethyl butyrate. The effect of temperature and pH on lipolytic activity, as measured by acid production, has also been investigated.

IN THE COURSE of studies on the stability of pharmaceutical products a group of bacteria possessing lipolytic activity was required. A preliminary survey was undertaken of all organisms available to us in the Department together with organisms (kindly supplied by Dr. E. I. Garvie of the National Institute for Research in Dairying) which originated from the National Collections of Type Cultures (NCTC), Industrial Bacteria (NCIB) and Dairy Organisms (NCDO) or were laboratory isolates. On the basis of this survey it was possible to select the following seven organisms for detailed study: *Serratia marcescens*, *Staphylococcus aureus*, *Micrococcus freudenreichii*, *Pseudomonas hydrophila*, *Achromobacter fischeri*, *Bacillus subtilis* and *Bacillus cereus*.

Of the above only the lipolytic activity of *Serr. marcescens* has been the subject of detailed investigation, especially by Tammisto (1933), who studied the hydrolysis of butterfat, ethyl butyrate and tributyrin by this organism. The optimal temperature was found to lie between 33° and 37° and the effect of pH on rate of hydrolysis showed a steady increase up to pH 10; for acetone dried preparations the optimal pH was 8·3–8·6.

Nashif & Nelson (1953*b*), working with centrifuged cultures of *Serr. marcescens* in borate buffers, found that the optimal pH for lipolysis was of the order of 8, and that the temperature optimum was 32°. Gorbach, Dedic & Lorenz (1954), using enzyme extracts, found that the optimal pH and temperature for the hydrolysis of butterfat by *Serr. marcescens* were 8–8·5 and 44°, respectively.

Serr. marcescens was included in the present study to attempt to clarify apparent differences reported in the above studies. References to the lipolytic activity of the remaining organisms is much less detailed and nomenclature is often imprecise.

Regarding the Bacillaceae, Tammisto (1933) stated that *B. subtilis* would decompose butterfat and ethyl laurate, and Goeters (1951) noted that members of the *B. mesentericus-subtilis* group were lipolytic. Smith, Lubert & Thornton (1949) showed that *B. subtilis* would not decompose tributyrin and Jones & Richards (1952) made a qualitative investigation of some aerobic sporeforming rods and noted that they were generally more active on tributyrin agar than on butterfat-night blue agar.

Much work has been directed towards investigating relationships between lipolytic activity and pathogenicity of the staphylococci. In this context it should be noted that, whereas the so-called *Staphylococcus albus* is not usually regarded as a human pathogen and has not therefore received much attention in this field, Davies (1954) reported that several strains of this organism possessed lipolytic activity when grown on a nile blue sulphate agar. Earlier Berry (1933) had also shown *Staph. albus* to be lipolytic.

MATERIALS AND METHODS

Cultures

The biochemical, morphological and cultural characteristics of the seven organisms studied were compared with organisms of similar name described in Bergey's *Manual* (Breed, Murray & Smith, 1957) except that in the case of the strain received as *Staph. albus* the classification as given by Shaw, Stitt & Cowan (1951) was adopted. With the following exceptions the organisms were found to be correctly named: *Achr. fischeri* matched *Photobacterium fischeri*, although in our hands it did not produce fluorescence in 3% sodium chloride broth and grew moderately at 37°. *Ps. hydrophila* corresponded to *Aeromonas hydrophila* (which is in accord with the observation of Rhodes, 1959) and gave positive methyl red and Voges-Proskauer reactions in a peptone medium—a medium which was not, however, of the same composition as that of Fouad & Richards recommended by Rhodes (1959). *Staph. albus* corresponded to *Staphylococcus* Rosenbach species (1)—*Staphylococcus aureus* Rosenbach emend. Shaw, Stitt & Cowan (1951)—and will be referred to as *Staph. aureus* hereafter. It should be noted that this strain gave only a weakly positive coagulase test with rabbit serum after 24 hr. In the case of the other two organisms the original name will be retained.

Qualitative detection of lipases

The laboratory stock of micro-organisms together with organisms from the National Institute for Research in Dairying were screened for lipolytic activity with olive oil, butterfat, triacetin, ethyl laurate, ethyl butyrate or coconut oil as substrates with the method of Jones & Richards (1952), employing victoria blue as an indicator.

The acid salt of victoria blue is blue and water soluble whereas the free base is red and fat soluble. When a medium at pH 8 containing victoria blue and a fat is shaken well in the molten state the dye is extracted into the fat as the red base. On pouring and allowing the agar to set, a pinkish medium is obtained. Lipolytic colonies grown on the medium will hydrolyze the fat, which results in the production of fatty acid and liberation of the dye. The fatty acid converts the dye to the acid salt again, and this appears as a blue zone around the colonies. The success of this method thus depends upon the use of water insoluble substrates.

The composition of the medium used was: peptone, 10 g; yeast extract (Difco), 3 g; sodium chloride, 5 g; agar, 20 g; aqueous solution of victoria blue (1 in 1,500), 100 ml; water to 1 l; final pH 7·8. The appropriate substrate was added in 5% concentration and the medium sterilized in 15 ml quantities by autoclaving at 115°

for 30 min. Before pouring the agar the substrate in it was dispersed by shaking. The plates were incubated at 37° for 2 hr to allow the pink colouration to develop, then stock cultures were streaked on to the plates which were incubated at 37° for 4 days. One set of plates containing butterfat was incubated at 25°. A positive lipolysis was recorded when the medium surrounding the colonies was blue on a pinkish background.

Activity towards tributyrin was tested with a medium similar to that described in Specification (1940). It contained: peptone, 5 g; yeast extract (Difco), 3 g; agar, 20 g; tributyrin, 10 g; water to 1 l; final pH 7·8. The tributyrin was dispersed with a hand homogenizer and the medium was sterilized by heating at 80° for 30 min on 3 successive days. Clear zones around colonies on this agar were taken as indicative of positive lipolysis.

It was found impossible to obtain the differential staining with victoria blue when ethyl butyrate and triacetin were the substrates used because of their solubility in the medium. To overcome this, nutrient broth was prepared and triacetin or ethyl butyrate, sterilized by filtration, was added to a final concentration of 1% (v/v). Aqueous 0·01% (w/v) bromothymol blue was incorporated to detect any acid production due to lipolysis. A positive reaction was recorded when the pH after incubation for 3 days at 37° was less than 6·2 as shown by a capillator. The results of these tests are summarized in Table 1.

Table 1. *The effect of substrate upon the lipolytic power of seven organisms*

Test organism	Reactions* on							
	Victoria blue agar containing						Bromothymol blue broth containing	
	Olive oil	Coconut oil	Ethyl laurate	Butter fat†	Butter fat‡	Tri-butyrin agar	Tri-acetin	Ethyl butyrate
Staph. aureus	++	++	+	++	++	++	++	0
Achr. fischeri	+	+	–	+	+	+	++	0
Ps. hydrophila	++	++	+	++	++	++	++	0
B. cereus	–	(+)	–	–	–	+	+	0
Serr. marcescens	++	++	+	++	++	++	–	0
Mic. freudenreichii	++	++	–	++	++	++	+	0
B. subtilis	–	–	–	–	(+)	++	++	–

* Reactions: ++, strong lipolysis; +, lipolysis; (+), weak lipolysis; –, no lipolysis; 0, no growth.
† Incubated at 25°. ‡ Incubated at 37°.

As reported by other workers, e.g. Jones & Richards (1952), it can be seen that the results of tests involving the use of victoria blue and the higher triglycerides do not completely correlate with those obtained with tributyrin agar which, in general, gave a higher number of lipolytic species. Coconut oil was found to give clearer positive results than did olive oil.

By means of this survey, seven organisms were selected for further study: *Staphylococcus albus* NCTC 189, *Micrococcus freudenreichii* NCDO 1223, *Achromobacter fischeri* NCIB 8008, *Pseudomonas hydrophila* NCTC 7810, *Bacillus subtilis* NCDO 736, *B. cereus* NCTC 5097 and *Serratia marcescens* PHI. The sporeformers were selected because the results obtained with them indicated that they might be more active against the simpler triglycerides, and esters, than the other bacteria studied.

Quantitative investigations on lipases

The method of Fiore & Nord (1949) was adopted with suitable modifications. The substrates investigated were olive oil, tributyrin, ethyl laurate and ethyl butyrate.

Growth of bacterial cultures. Nutrient broth (peptone, 5 g; Lab-Lemco, 5 g; sodium chloride, 5 g; water to 1 l; final pH 7·2) was prepared and distributed into 500 ml narrow necked conical flasks in 250 ml quantities and sterilized by heating at 115° for 30 min. These flasks were inoculated with 8 ml of an overnight culture of the organism, placed on an apparatus designed to rotate the flasks at an angle of 75° about their vertical axes at 20 rev/min and incubated for 10 hr at the optimal growth temperature of the bacteria (previously determined). The concentration of bacteria was determined by measuring the opacity of the culture nephelometrically and reading off the dry weight of bacteria/ml from calibration curves previously constructed. For the optimal lipolytic temperature experiments the bacteria were grown in 12 ml quantities of broth in 25 ml tubes placed on the rotator.

Preparation of substrates. Volumes of 10 ml of the appropriate substrate (sterilized by filtration) were placed in a sterile vortex beaker of an homogenizer (Measuring and Scientific Equipment Ltd., Spenser St., London S.W.1) and 90 ml of sterile emulgent added. For olive oil, a 0·75% (w/v) tragacanth mucilage was found to give the best results, whereas for the other substrates 4% (w/v) polyvinyl alcohol (B.D.H.) in water was used. The mixture was homogenized for 10 min at the same speed to produce similar emulsions each time. Schonheyder & Volquvartz (1945) showed that lipolysis depended at least in part upon the degree of emulsification achieved. The homogenization was performed under a screen to minimize contamination.

Lipase determination. Volumes of 5 ml of the emulsified substrate were pipetted into 30 ml sterile screwcap bottles, together with 5 ml of the appropriate buffer at a concentration of 0·2 M, 2·5 ml of bacterial culture, 2·5 ml of sterile water, and sterile glass beads. The bottles were packed into a basket designed to oscillate in a water bath with a frequency of 98 oscillations/min and an amplitude of 2 in. The rate of oscillation was kept constant since Trussell & Weed (1937), Mattick & Kay (1938) and others have shown that the degree of shaking affects the extent of lipolysis; this finding was confirmed. In our experiments the bottles were shaken at 37° for 24 hr in the water bath, removed and 15 ml of a mixture of equal parts of alcohol and acetone added to stop the reaction, to split the emulsion and to precipitate the emulgent. After standing for 10 min the mixtures were filtered through Whatman no. 1 paper into dry flasks, and 10 ml aliquots of filtrate were then titrated with 0·04 N aqueous sodium hydroxide solution using phenolphthalein as indicator. A control experiment was always performed with cultures autoclaved to destroy enzymic activity. The difference was taken as the amount of acid produced by the bacteria. The results were calculated as the number of ml of 0·02 N acid liberated/mg of bacteria in 24 hr.

To study the effect of pH upon lipase activity 5 ml of the required buffer were placed in the reaction bottles. The following buffer systems were used: phosphate-citric acid (McIlvaine); disodium-monosodium phosphate (Sørensen); tris(hydroxymethyl)aminomethane-hydrochloric acid (tris); and glycine-sodium hydroxide. A pH range of 3–11 with appropriate overlaps was thus covered. Borate buffers were not used because of their reported toxicity (Jeseski, Halvorson & Macy, 1950). McIlvaine and glycine-sodium hydroxide buffers were sterilized by filtration, and the others by autoclaving at 115° for 20 min.

To establish that the buffering capacity of the experimental systems used was adequate, the pH's of the reaction mixtures were measured by means of narrow range indicator papers. With tributyrin as substrate and with the seven selected bacterial species, no measurable change in pH could be detected.

The effect of temperature upon lipolysis was measured by incubating cultures in Sørensen buffer (pH 6·9) at temperatures ranging from 20° to 42°.

Under the experimental conditions used, there was no detectable spontaneous hydrolysis of the substrates.

RESULTS

The means of two experiments to determine the optimal pH are summarized in Table 2, and those determining optimal temperature in Table 3. Satisfactory agreement was obtained between duplicate experiments.

Table 2. *The effect of* pH, *substrate, and type of buffer on the lipolytic activity of seven organisms*

Test organism	Substrate*	Vol (ml) of 0·02 N acid liberated at pH†											
		5·1‡	6·1‡	7·2‡	8·0‡	5·6*	6·9*	7·9*	7·3**	8·1**	9·2**	9·8††	10·6††
Ps. hydrophila	a	−1·4	4·9	11·6	12·7	1·4	12·7	15·9	4·3	6·5	10·1	2·7	2·9
	b	5·1	7·6	11·6	15·5	7·6	14·0	15·4	6·1	10·8	17·5	8·7	12·5
	c	0·0	0·9	6·9	8·3	1·4	7·3	8·3	0·5	4·8	4·2	2·7	2·7
	d	0·0	1·4	0·4	1·6	0·4	2·0	0·5	0	1·4	2·2	2·5	6·0
B. subtilis	a	−2·1	−13·0	8·2	1·0	0·5	−1·7	1·4	0·2	3·1	7·5	10·1	5·3
	b	−1·0	3·8	10·5	8·1	5·5	39·9	6·2	2·2	20·7	9·1	2·6	6·5
	c	−10·6	−9·9	−2·1	5·7	1·0	3·6	7·9	−1·2	1·4	10·9	3·8	1·6
	d	−1·0	−1·5	0·9	3·8	1·1	1·5	3·3	2·8	1·3	6·4	7·9	4·8
B. cereus	a	−9·9	−17·5	−14·5	2·1	−2·1	−0·5	6·2	−0·5	5·5	10·6	8·1	6·7
	b	−2·5	29·3	127·4	163·1	19·4	68·1	108·0	3·5	32·5	51·0	18·9	16·8
	c	−6·1	−9·7	−3·2	11·1	−0·3	3·5	14·3	0·3	8·3	12·7	11·5	7·0
	d	0·5	4·8	−0·5	3·0	0·9	1·4	3·5	1·4	4·4	6·5	6·7	10·4
Achr. fischeri	a	−3·3	−6·0	−8·6	1·8	−1·6	6·2	7·5	−2·8	3·8	8·4	5·9	5·2
	b	0·4	28·8	76·9	114·0	2·6	50·0	63·2	1·9	19·4	35·6	10·5	6·0
	c	−2·0	−6·5	0·1	2·6	−2·5	−0·3	4·6	−1·6	3·6	4·9	6·0	5·8
	d	−0·3	−1·0	−0·4	1·9	−0·41	−0·82	1·5	0·0	−1·5	1·6	0·0	0·0
Staph. aureus	a	13·8	22·4	31·1	27·5	N	N	N	13·4	15·5	12·0	10·6	7·2
	b	26·1	80·1	132·0	123·9	N	N	N	36·7	47·3	45·9	12·2	6·0
	c	4·2	7·1	12·4	10·5	N	N	N	9·9	7·6	6·3	4·2	1·4
	d	1·8	3·2	8·0	4·2	N	N	N	3·5	2·1	2·1	1·4	1·1
Serr. marcescens	a	−6·3	−2·3	2·0	11·3	N	N	N	6·2	9·0	10·5	3·2	5·6
	b	−0·7	−1·2	2·8	7·3	N	N	N	2·0	6·0	8·7	3·0	4·9
	c	N	−0·6	1·7	6·8	N	N	N	5·2	9·1	11·6	6·8	8·9
	d	N	0·4	1·5	4·0	N	N	N	1·7	1·3	2·4	3·6	7·3
Mic. freundenreichii	a	0·3	8·4	8·4	7·2	N	N	N	3·8	5·3	4·6	3·1	0·9
	b	2·6	7·4	11·3	11·7	N	N	N	7·2	9·8	8·7	7·4	6·9
	c	0·2	0·2	N	6·9	N	N	N	2·7	2·7	3·9	5·7	5·1
	d	0·8	1·2	1·8	2·1	N	N	N	0·6	1·8	2·0	2·0	1·8

* Substrates: a, olive oil; b, tributyrin; c, ethyl laurate; d, ethyl butyrate.

† Acid liberated by 1 mg (dry wt) of organisms in 24 hr.

‡ pH maintained by McIlvaine citrate phosphate buffer. § pH maintained by Sørensen buffer. ** pH maintained by tris buffer. †† pH maintained by glycine-sodium hydroxide buffer. N, experiments not done.

The effect of pH on lipase activity

Staph. aureus generally had an optimal pH of 6·5–7·5 with all four substrates (Fig. 1c shows the results obtained with ethyl laurate and olive oil). *Mic. freudenreichii* had a pH optimum of 6–7 for olive oil and tributyrin with McIlvaine buffer and pH 8 with tris buffer; for ethyl laurate and ethyl butyrate, however, the optimal pH was 7·5–8·1 with both buffers.

The general shape of the pH curves obtained with *Serr. marcescens* and *Ps. hydrophila* with McIlvaine, glycine and tris buffers tended to give no characteristic optimum for all substrates even with values as high as pH 10·6. The activity of *Serr. marcescens* lipase on olive oil is illustrated in Fig. 1b. Both these organisms utilize citrate as sole carbon source as determined by growing them in Koser's citrate medium; this fact is reflected in the experiment illustrated by Fig. 1b where the citrate from the McIlvaine buffer was obviously utilized, giving rise to apparent negative acid production.

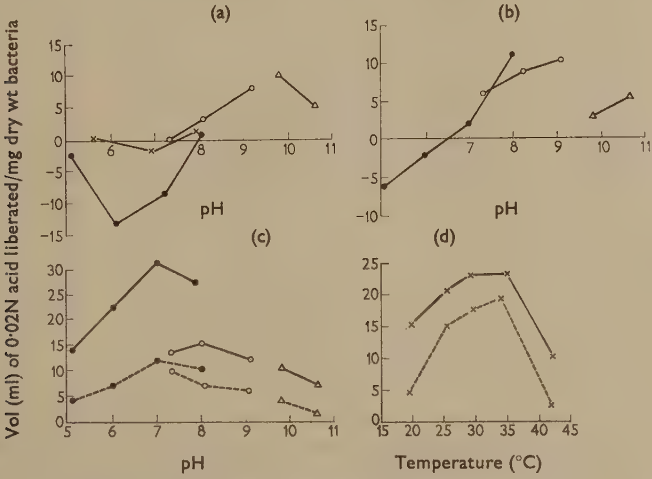

Fig. 1. The effect of pH and temperature on the lipolytic activity of four organisms.
Continuous lines, olive oil; dashes, ethyl laurate.
Full circles, McIlvaine buffer; open circles, tris buffer; crosses, Sørensen buffer; triangles, glycine-sodium hydroxide buffer.
(a) *B. subtilis*; (b) and (d) *Serr. marcescens*; (c) *Staph. aureus*;

Achr. fischeri lipase had an optimal pH lying between 8·5 and 9·5. A similar result was obtained with *B. cereus* except when ethyl butyrate was the substrate, in which case no optimum was observed up to pH 10·6.

B. subtilis lipase had a pH optimum of 8·5–9·5 with olive oil, ethyl butyrate and ethyl laurate. With tributyrin, the optimal value was pH 6·5–7·5 with Sørensen buffer and 7·5–8·5 with tris buffer. *B. subtilis* and *B. cereus* also utilize citrate readily as can be seen from Fig. 1a: McIlvaine's buffer gave an apparent negative acid production over nearly the whole range of pH values tested.

There is some evidence from the curves in Fig. 1 that the buffers used were exerting an independent action on lipolytic activity. Thus Fig. 1b shows glycine buffer to be inhibitory to *Serratia* lipase, compared with tris buffer. Similarly in Fig. 1c tris buffer appears to have depressed the activity of staphylococcal lipase. if considered in relation to the result with McIlvaine buffer.

The effect of temperature on lipase activity

The optimal temperature for olive oil and ethyl laurate lipolysis for all micro-organisms was in the range 32°–37° (Table 3 and Fig. 1d). It is interesting to note that even at 20° and 42° lipolytic activity persisted. It was thought of interest to measure the viability of organisms during some of the experimental procedures described above. Accordingly viable counts were performed by serial dilution and plating from a replicate bottle in the lipase determination using olive oil and McIlvaine phosphate citrate buffer as described above. The results are shown in Table 4. The general trend of these results shows that for *Staph. aureus*, *Serr. marcescens* and *B. cereus* there was an appreciable fall in viable numbers during the

Table 3. *The effect of temperature on the breakdown of olive oil and ethyl laurate by seven organisms at* pH 6·9

Test organism	Substrate*	Vol. (ml) of 0·02 N acid liberated from substrates at (°C)†				
		19·5°	25·5°	29·0°	34·0°	42·0°
Mic. freudenreichii	a	4·9	N	24·9	23·6	9·4
	b	0·2	N	0·6	1·3	1·3
B. cereus	a	1·7	4·1	6·4	15·6	6·0
	b	0·0	0·75	1·5	3·3	3·0
Achr. fischeri	a	2·8	1·9	2·7	9·5	2·8
	b	0·9	0·0	1·1	5·6	4·0
Serr. marcescens	a	15·2	20·8	23·0	23·5	10·2
	b	4·5	15·0	17·7	19·3	2·6
Ps. hydrophila	a	7·0	8·7	12·0	23·0	13·4
	b	0·9	0·9	1·3	9·5	4·3
Staph. aureus	a	1·8	5·3	3·2	24·9	8·5
	b	0·3	0·3	0·5	14·1	6·6
B. subtilis	a	2·6	6·2	3·5	17·8	6·1
	b	0·7	0·7	1·4	11·3	2·9

* Substrates: a, olive oil; b, ethyl laurate. † Acid liberated by 1 mg (dry wt) of organisms in 24 hr. N, experiments not done.

Table 4. *The change in viable numbers of four organisms during lipolysis of olive oil*

Test organism	Viable count (10^6/ml) after (hr)		
	0	16	24
Staph. aureus	310	100	100
Serr. marcescens	900	120	680
B. cereus	65	10	80
Achr. fischeri	22	68	270

first 16 hr. After this *Achr. fischeri*, *Serr. marcescens* and *B. cereus* showed a marked increase during the next 8 hr, whereas *Staph. aureus* showed no increase. This phenomenon may be connected with the fact that *Achr. fischeri*, *Serr. marcescens* and *B. cereus* are able to utilize citrate (present in the buffer) for growth while *Staph. aureus* cannot. The exact nature of the inhibitory or lethal effect shown during the first 16 hr is not readily explicable, subsequent growth in the case of the three organisms showing that it may be due to a process of adaptation to the environment and substrates available.

With a view to throwing some light upon the resistance of the lipases of *Serr. marcescens* and *Ps. hydrophila* to alkaline conditions (a feature which distinguishes these two organisms from the other five studied), the effect of pH upon their growth was investigated. Volumes of media were prepared by mixing equal volumes of double strength nutrient broth and appropriate buffer (0·2 M). These mixtures were distributed in 10 ml amounts and sterilized by heating at 115° for 30 min. The final pH was checked with narrow range indicator papers.

Table 5. *The effect of* pH *upon the growth of seven organisms at their optimal temperature*

Test organism and growth temperature (°C)	Relative nephelometer reading after 10 hr at pH						
	5·9*	7·0*	7·6*	8·0†	8·8†	9·0‡	10·6‡
Staph. aureus § (37°)	31	36	20	24	17	24	0
Serr. marcescens (30°)	88	88	51	72	43	59	14
Mic. freudenreichii (30°)	N	44	48	40	36	41	0
Ps. hydrophila (30°)	N	46	34	36	29	34	2
B. subtilis § (30°)	N	24	23	31	26	31	0
B. cereus § (30°)	N	26	12	30	31	27	0
Achr. fischeri (30°)	38	14	16	28	23	22	0

* pH maintained by Sørensen buffer. † pH maintained by tris buffer. ‡ pH maintained by glycine-sodium hydroxide buffer.

§ The nephelometer readings were difficult with these cultures due to granular growth; different pH's appeared to affect the degree of pellicle formation.

N, experiments not done.

An overnight culture was inoculated (0·1 ml) into each tube and the nephelometer reading recorded at the beginning of the experiment. The bacteria were then incubated on a rotator for 10 hr at their optimal growth temperature. The nephelometer reading was again recorded, and the difference taken to be a measure of the bacterial growth.

The results, summarized in Table 5, show that *Serr. marcescens* and *Ps. hydrophila* grew well at pH 9·0 and poorly at pH 10·6. The others grew well quite at pH 9·0 but not at all at pH 10·6.

DISCUSSION

The terms lipase and esterase have been used somewhat ambiguously by various workers. Nachlas & Seligman (1949) have commented on this. Strictly speaking a lipase is an enzyme which will hydrolyze triglycerides, and an esterase one which will hydrolyze simple esters (i.e. those of general formula R.COO.R′). However, as can be seen by the results in this paper, both types of substrate are attacked. Whereas most of the organisms attacked the esters poorly, *B. cereus* and *B. subtilis* attacked olive oil and ethyl butyrate with approximately equal activity. The evidence of optimal pH and temperature would appear to preclude the idea of two enzymes acting in these cases. There would seem to be one enzyme having low specificity towards triglycerides and simple esters. The term lipase is used in this paper with this latter interpretation in mind.

There are so many factors which can affect the quantitative results that experimental conditions must be clearly stated and maintained. Some of these factors will be considered.

The temperature at which the organisms are grown has a considerable effect upon the quantity of lipase produced (see the work of Nashif & Nelson (1953*a*) on *Ps. fragi*). Throughout our experiments, the bacteria were grown at their respective optimal growth temperatures at pH 7, although it does not follow that this would be the optimal temperature for lipase production. Nashif & Nelson (1953*a*) and others have shown that the quantity of lipase produced may also be affected by the degree of aeration. and the surface area and pH of the growth medium.

In the lipase determinations the globule size of the emulsion and the degree of shaking can alter the amount of acid liberated, as shown by Trussel & Weed (1937), Mattick & Kay (1938). Schonheyder & Volquvartz (1945). and confirmed in our preliminary work. In a heterogeneous system the reaction will depend upon the available surface of the substrate. and thus the smaller the globules the greater will be the rate of hydrolysis up to a given limiting rate for each system studied. Shaking the reaction mixture will also tend to increase the effective surface. It will also increase aeration of the liquid and prevent localized build up of acid which might have a toxic effect on the enzymes. Our preliminary experiments also confirmed this in that the rate of hydrolysis increased with degree of shaking up to a point beyond which the rate decreased slightly.

The terms optimal pH and optimal temperature are only relative. and need definition. In this work the optimal pH is taken to be that pH at which the greatest amount of lipolysis occurred in the system in 24 hr at 37°. The optimal temperature is that temperature at which the maximal lipolysis occurred in 24 hr at pH 6·9. The optimal pH value will result from a combination of factors. some of which are briefly as follows: the effect of pH on the stability of the enzyme, on the velocity of enzyme-substrate combination and breakdown. on the affinities of enzyme and substrate for each other (in this case nullified by the use of a large excess of substrate), on the equilibrium point of the reaction, and on the substrate (although this factor is negligible in these experiments as the esters are not ionized). Similar factors are operative in determining the optimal temperature for lipolysis. From the relevant Tables it can be seen that the optimal lipolysis temperature and optimal lipolysis pH do not completely correspond with the optimal pH and temperature for growth. In the cases of *Serr. marcescens* and *Ps. hydrophila* activity increased with pH up to 10·6. The lipases of these two organisms appear to be quite resistant to this pH. It is worthy of note that both organisms will grow, albeit poorly, at pH 10·6.

The qualitative results show, too, that the degree of lipolysis varied with the nature of the substrate. In general, the triglycerides were more readily attacked than the simple esters ethyl laurate and ethyl butyrate. As one would expect, the lower triglycerides were readily hydrolyzed, although *Serr. marcescens* hydrolyzed olive oil more readily than tributyrin and triacetin. A large number of the organisms tested hydrolyzed tributyrin and triacetin, leaving the other substrates unattacked. Tributyrin agar is thus not a very good medium for lipase screening because of its low selectivity.

For olive oil and tributyrin, the agreement between qualitative and quantitative results was good whereas for the other two substrates it was poor. Although the preformed lipases of all seven organisms would hydrolyze ethyl laurate and ethyl butyrate, only *Staph. aureus*, *Serr. marcescens* and *Ps. hydrophila* gave a positive lipolysis on the victoria blue-ethyl laurate medium, the others being negative. *B. subtilis* was the only organism which would grow in ethyl butyrate broth, but did not show lipolysis.

The work reported in this paper with *Serr. marcescens* confirms the work of Tammisto (1933) on the effect of pH upon lipolytic activity and the optimal lipolysis temperature of whole cells. The findings of Gorbach, Dedic & Lorenz (1954), who tested enzyme extracts on butterfat, of Nashif & Nelson (1953*b*) who used centrifuged cultures, and of Tammisto (1933), who studied a cell free preparation, are consistent with the hypothesis that the enzyme is extracellular and has a pH optimum lower than that for the whole cells.

REFERENCES

BERRY, J. A. (1933). The determination of microbial lipase by copper soap formation. *J. Bact.* **25**, 433.

BREED, R. S., MURRAY, E. D. G. & SMITH, N. R. (1957). Bergey's *Manual of Determinative Bacteriology*, 7th ed. London: Baillière, Tindall & Cox.

DAVIES, M. E. (1954). Study of the diffusible lipase produced by staphylococci and of its immunological properties. *J. gen. Microbiol.* **11**, 37.

FIORE, V. & NORD, F. F. (1949). The determination of lipase activity with the aid of polyvinyl alcohol. *Arch. Biochem.* **23**, 473.

GOETERS, W. (1951). Microbiotic fat metabolism in the alimentary canal of infants and children. *Z. Kinderheilk.* **69**, 438.

GORBACH, G., DEDIC, C. & LORENZ, H. (1954). The enrichment and detection of bacterial lipases. *Arch. Microbiol.* **21**, 273.

JESESKI, J. J., HALVORSON, H. O. & MACY, H. (1950). Action of microorganisms on fat. *J. Bact.* **59**, 641.

JONES, A. & RICHARDS, T. (1952). Night blue and victoria blue as indicators in lipolysis media. *Proc. Soc. appl. Bact.* **15**, 82.

MATTICK, E. C. V. & KAY, H. D. (1938). A lipase of cows' milk. *J. Dairy Res.* **9**, 58.

NACHLAS, M. M. & SELIGMAN, A. M. (1949). Evidence for the specificity of esterase and lipase by the use of three chromogenic substances. *J. biol. Chem.* **181**, 343.

NASHIF, S. A. & NELSON, F. E. (1953*a*). The lipases of *Pseudomonas fragi*. II. Factors affecting lipase production. *J. Dairy Sci.* **36**, 471.

NASHIF, S. A. & NELSON, F. E. (1953*b*). The extracellular lipases of some Gram-negative non-sporeforming rod-shaped bacteria. *J. Dairy Sci.* **36**, 698.

Rhodes, M. E. (1959). The characterisation of *Pseudomonas fluorescens*. *J. gen. Microbiol.* **21**, 221.

Schonheyder, F. & Volqvartz, H. (1945). Affinity of pig pancreas lipase for tricaproin in heterogeneous solution. *Acta physiol. scand.* **9**, 57.

Shaw, C., Stitt, J. M. & Cowan, S. T. (1951). Staphylococci and their classification. *J. gen. Microbiol.* **5**, 1010.

Smith, L. M., Lubert, D. J. & Thornton, H. R. (1949). Estimation of lipase in dairy products: III. Lipase activity in cultures of micro-organisms and in cheese. *Canad. J. Res.* **27F**, 499.

Specification (1940). British Standard Method for the Microbiological Examination of Butter, no. 895. London: British Standards Institute.

Tammisto, E. S. (1933). The lipases of bacteria. *Ann. Acad. Sci. Fenn.* A38, no. 5.

Trussel, R. E. & Weed, L. A. (1937). The lipolytic activity of staphylococci on some pure triglycerides. *J. Bact.* **33**, 381.

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SMITH, W. K. (1962). *J. appl. Bact.* **25** (1), 83–86.

A MECHANICAL SOIL PERCOLATOR

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SUMMARY: A mechanically operated soil percolator is described. Each unit of a battery is independent. Anaerobic percolation is possible.

THE VALUE of the soil percolation technique introduced by Lees & Quastel (1944) is attested by the variety of modified percolators subsequently devised (Audus, 1946; Lees, 1949, 1950; Temple, 1951; Jefferys & Smith, 1951; Collins & Sims, 1956; Bryner & Anderson, 1957; Gundersen, 1960). This number is probably an indication of the difficulties encountered in operating a battery of percolators from a common suction or pressure manifold. Generally the operating pressure has to be kept within fairly close limits to avoid complete cessation of percolation, flooding or other malfunction. A change in the resistance of one percolator is usually reflected by disturbances in the flow through the remainder of the battery, and fluctuations in the water supply or air line pressure can have disastrous results.

The apparatus described here was devised to overcome these defects by making the percolators independent of each other, and by employing a mechanical actuating system. This has been achieved by making each percolator in the form of a rotating circular tube. As the unit revolves the soil is, in effect, passed through the pool of percolating solution lying at the lowest point of the circle and this pool of liquid acts as a piston to draw air through the retreating soil. This simple system would, of course, circulate the same air through the soil again and again. To prevent this a valve has been incorporated, so that air is expired after passing through the soil and fresh air is drawn in to take its place.

MATERIALS AND METHODS

Percolator units. Each unit consists of a soil tube, a sampling port and a valve connected by tubing as indicated in Fig. 1a. The soil tube should be curved to the radius of the unit and tapered to allow complete drainage of the percolating solution. The dimensions can be varied to suit experimental requirements. The sampling port is a simple T piece with a stopper in the side arm. The valve is illustrated in Fig. 1b. It is operated by a $\frac{1}{2}$ in. stainless steel ball bearing which seats itself at one or other end of the tube in which it is contained. Smaller bearings are not heavy enough to give a good seal. The blister in the tube delays the ball (for about 15 sec at 45 rev/hr) to allow the percolating solution to pass through the valve. The air inlet and outlet tubes must be long enough to remain above the liquid level as the unit revolves; cranking each towards the centre helps to maintain this head. The traps are necessary to retain the small amount of liquid that drains on each revolution. Any suitable tubing may be used to connect these three components; $\frac{3}{8}$ in. bore transparent P.V.C. tubing proved satisfactory.

The units are fastened by clips on each side of discs of $\frac{1}{2}$ in. plywood. In the model illustrated (Plate 1) four such discs, 2 ft in diameter and capable of carrying 8 percolators, are mounted on a common shaft. Portions of the discs are cut away to reduce the weight of the assembly and to facilitate access to the inner percolators.

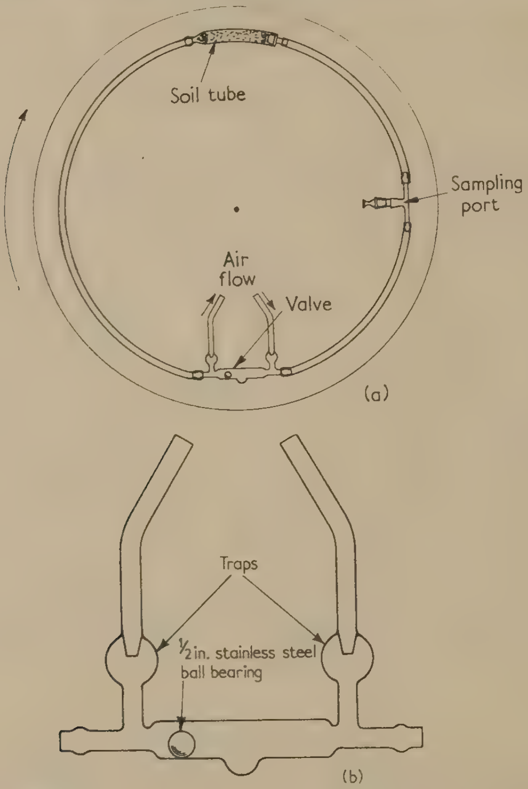

Fig. 1. Diagram of the percolator unit (*a*), with details of the valve (*b*).

The battery is driven by a fractional horsepower electric motor geared to give one revolution in approximately 80 sec (45 rev/hr). Other speeds may be more suitable for different experimental conditions. Since the discs can be balanced quite easily the power required to drive the unit at this slow speed is very low.

Operation. As the apparatus and experimental procedure are susceptible to many variations to suit particular purposes only a brief outline need be given here. The soil tube is packed with a layer of glass wool or other suitable support, soil granules and another layer of the support. Because the operating pressure is only a few inches head of water, the soil column should not be very deep and it may be desirable to add sand or a similar diluent to prevent the clogging which occurs with some soils. The percolator unit is assembled and clipped to the disc. Water to moisten the soil or percolating solution may then be added. conveniently through the soil tube; percolation is then started.

Experimental. The investigation for which this percolator was devised was discontinued at an early stage and few results were obtained. In the course of development of the apparatus the oxidation of ammonium was used as a measure of the effectiveness of the system. The data presented in Table 1 should be regarded as the results of a qualitative test of the percolators rather than a quantitative investigation of nitrification.

In this experiment 4 percolators were set up. Each contained 10 g of dry granules obtained by sieving used potting compost between 8 mm and 2 mm sieves. The soils were moistened by percolating with water which was drained off: then 30 ml of 0·1% $(NH_4)_2HPO_4$ solution were added to each percolator. Units 1 and 2, which had been flushed out with a mixture of nitrogen and carbon dioxide, were operated anaerobically by 'short-circuiting' the air inlet and outlet tubes. Percolation, at 1 rev/80 sec, was started and samples of percolating solution were withdrawn at intervals. Usually these were clear enough to permit estimations of ammonium by Nesslerization of appropriate dilutions. A glass electrode was used to determine pH values. The presence of nitrite was detected by a spot test with Griess-Ilosvay reagents.

Results and Discussion

The course of the experiment is outlined in Table 1. The initial ammonium concentration (actually determined on a sample withdrawn after percolation for 10 min

Table 1. *The results of four percolation experiments with $(NH_4)_2HPO_4$ solutions*

Total time (days)	Materials added* during experiments	Time after additions of $(NH_4)_3PO_4$ (hr)	Concentration† of ammonium N_2 (μg/ml of percolate) in percolator no.			
			1‡	2‡	3§	4§
0	$(NH_4)_2HPO_4$, 0·1%	0	110	112	105	100
1		22	91 (+)	92 (+)	91 (−)	81 (−)
8		196	88 (5·20)	100 (5·00)	36 (4·35)	108 (4·65)
8	$(NH_4)_2HPO_4$, 0·05%	0	77	77	58	87
9		19	71	76	53	82
10	M $NaHCO_3$	43	59 (6·80)	58 (6·80)	42 (5·80)	58 (7·00)
11		67	57	56	28	56
14	$CaCO_3$	139	78	84	0	0
14	$(NH_4)_2HPO_4$, 0·05%	2	75	66	66	63
14		4	66	62	38	31
15	$CaCO_3$	28	72	67	**	17
16		54	75	73	c. 10	10

* The percolators were charged with, or drained and recharged with, 30 ml of the $(NH_4)_2HPO_4$ solutions; volumes of 0·3 ml of $NaHCO_3$ and pinches of $CaCO_3$ were added.

† The symbols in brackets indicate the presence (+) and absence (−) of nitrite. and the numbers in brackets are the pH values obtained.

‡ Percolators maintained under anaerobic conditions.

§ Percolators maintained under aerobic conditions.

** Not tested because of turbidity.

to allow equilibration) was some 20% below the value calculated from the assay of the solution added and the amount of water retained by the soil. This, presumably, was due to adsorption of ammonium by the soil. The drop in the next 22 hr probably represents oxidation, since nitrite could be detected in the anaerobic percolators, in which the process appeared to have remained stationary at this stage. In percolator 3, the removal of ammonium proceeded further despite a fall in the pH value to 4·35.

At 196 hr the percolators were drained and fresh 0·05% $(NH_4)_2HPO_4$ solution was added. Little change in the ammonium concentration occurred until a small amount of $NaHCO_3$ was added. This was followed by a drop in the ammonium concentration to levels which remained steady (except in percolator 3) until a pinch of chalk was added to each percolator. Thereafter the ammonium disappeared from the aerobic percolators, whereas the concentrations in the anaerobic percolating solutions rose again. This increase may have been due to displacement of adsorbed ammonium ions from the soil colloids by calcium ions. In the aerobic percolators the ammonium had almost disappeared in less than 3 days, but the concentration in the anaerobic units was unchanged after 8 days.

Once the components of the rotating percolator have been made it is easy to set up and reliable in operation. The data reported above indicate that soil processes such as nitrification proceed with reasonable speed. These processes, and the effects of such variables as restricted aeration and $CaCO_3$ addition, can be easily followed.

The author is indebted to Mr. G. Merkins and others for the construction of the percolator and to Mr. W. van de Bospoort and Mr. R. Gee for making the valves and other glass parts.

REFERENCES

AUDUS, L. J. (1946). A new soil perfusion apparatus. *Nature, Lond.*, **158**, 419.

BRYNER, L. C. & ANDERSON, R. (1957). Microorganisms in leaching sulfide minerals. *Industr. Engng Chem.* (*Industr.*) **49**, 1721.

COLLINS, F. M. & SIMS, C. M. (1956). A compact soil perfusion apparatus. *Nature, Lond.*, **178**, 1073.

GUNDERSEN, K. (1960). An all-round soil percolator. *Science* **132**, 224.

JEFFERYS, E. G. & SMITH, W. K. (1951). A new type of soil percolator. *Proc. Soc. appl. Bact.* **14**, 169.

LEES, H. (1949). The soil percolation technique. *Plant & Soil* **1**, 221.

LEES, H. (1950). A percolating respirometer. *Nature, Lond.*, **166**, 118.

LEES, H. & QUASTEL, J. H. (1944). A new technique for the study of soil sterilisation. *Chem. & Ind.* **22**, 238.

TEMPLE, K. L. (1951). A modified design of the Lees soil percolation apparatus. *Soil Sci.* **71**, 209.

EXPLANATION OF PLATE

A rotary soil percolator capable of carrying eight percolator units of the type fitted to one side of one of the four plywood discs.

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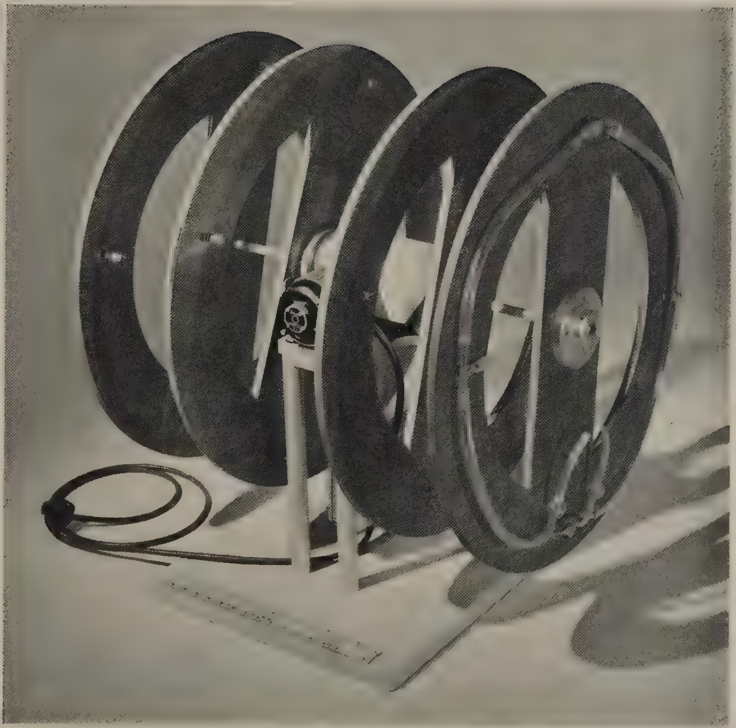

W. K. SMITH — A MECHANICAL SOIL PERCOLATOR. PLATE 1

Geldreich, E. E., Huff, C. B., Bordner, R. H., Kabler, P. W. & Clark, H. F. (1962). *J. appl. Bact.* **25** (1), 87–93.

THE FAECAL COLI-AEROGENES FLORA OF SOILS FROM VARIOUS GEOGRAPHICAL AREAS

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SUMMARY: A study has been made of coli-aerogenes bacteria isolated from 251 soil samples collected from 26 States and 3 foreign countries. Based on land use, the soils were classified in 8 classes of the undisturbed group and one additional group of soils which were known to have been exposed to various kinds of faecal pollution.

Faecal coli-aerogenes organisms were usually absent, or present in comparatively small numbers only, in undisturbed soils, with most counts being less than 1·8/g. There was a marked increase in the numbers in soils of the polluted group, with a density of between 3,300 and 49,000/g.

Intermediate types represented 76% of the 2,348 strains isolated from the undisturbed soil classes, as contrasted with only 17% of 665 polluted soil strains. The IMViC reactions of the predominant type from the undisturbed group were − + − +, and this type represented 48% of the 2,348 strains isolated. The sanitary significance of the − + − + intermediate type as a soil coli-aerogenes organism, in relation to data on types from faecal samples and certain remote mountain streams, suggests that the strains giving these IMViC reactions, and which are 45° negative, may possibly be considered as a type characteristic in unpolluted soils but relatively infrequent where faecal pollution has occurred.

Since soil contains high numbers of organisms, other than coli-aerogenes bacteria, which frequently produce false positive reactions by the 'confirmed test' procedure, the 'completed test' method is recommended for the examination of soils for coli-aerogenes organisms.

Soil may contribute large numbers of bacteria to drainage water. The number and species present depend on such factors as soil type, erosion, land use and amount of rainfall. The possibility that soil may add to the coli-aerogenes content of water has been a subject of considerable interest and speculation in sanitary bacteriology. There still exists much controversy concerning the distribution, origin and sanitary significance of these bacteria.

Johnson & Levine (1917), Chen & Rettger (1920), Koser (1926), Raghavachari (1926) and Frank & Skinner (1941) have reported *Aerobacter aerogenes* to be the predominant coli-aerogenes type in soils, with percentages varying from 45·0 to 93·5. The intermediate type giving IMViC reactions of − + − + was found by Bardsley (1934) to be dominant in soil. Taylor (1951) considered *Bacterium coli* type I the most prevalent type regardless of location or soil type. Randall (1956) stated that the proportion of *B. coli* type I was directly related to the degree of probable pollution of the soil, and that the proportion of intermediates increased with remoteness of the soil from potential polluting agents.

All coli-aerogenes bacteria found in soil were thought by Parr (1939) to be of faecal origin. Griffin & Stuart (1940) suggested that only *Escherichia coli* was derived from faeces. Taylor (1951) found insufficient evidence to conclude that any of the coli-aerogenes types studied by him were normal soil inhabitants. It is also suggested in *Standard Methods for the Examination of Water and Wastewater* that coli-aerogenes bacteria (designated as the 'coliform group' in the United States) are probably not normal inhabitants of soil. It is assumed that these authors are considering only the coli-aerogenes group as defined in the practice of sanitary bacteriology and not the many bacteria that resemble these organisms but which are unable to grow at 35°.

These conflicting observations concerning the distribution and origin of coli-aerogenes types may in part be a result of variability of soil types and their associated environments. In addition, technical laboratory procedures have varied between enrichment in broth to direct streaks on selective agar media, between glucose to lactose fermentation, and between incubation at 30° to 44°. As a consequence, the resulting type distribution pattern would be influenced by whatever segment of the coli-aerogenes population might be favoured by a particular growth and identification procedure.

Moreover, practically all the investigations have depended upon the primary isolation of coli-aerogenes bacteria in liquid media: but such a procedure is not applicable to the quantitative estimation of various types of these organisms, as overgrowth by one or more types results in the suppression of minority organisms.

Recently the membrane filter technique for the study of the distribution patterns of coli-aerogenes organisms and the elevated temperature test for the detection of the faecal types have been used by Clark *et al.* (1957) and Geldreich *et al.* (1958, 1962). These studies of the distribution of coli-aerogenes bacteria in water and faecal samples suggested that the two procedures may offer an improved method for soil investigation and avoid past deficiencies in procedure.

MATERIALS AND METHODS

Sources and types of samples. In this investigation, 251 soils from various geographical areas in 26 States and three foreign countries were examined; these were grouped in the following classes: arid soils, which included samples from sand dunes, an alpine area and a salt flat; subterranean soils, consisting of samples from caves and a coal mine; submerged soils, collected from creek, river and lake bottoms and sludge deposits, and a submerged salt water beach soil; pasture land samples, from mountain pastures, sagebrush and mesquite open ranges, mesas, prairie land, blue grass field and farm livestock pastures; shore-line samples, collected from soil adjacent to various creeks, rivers, reservoirs, lakes and oceans and including also samples from swamps, bogs and marshlands; woodland samples, taken from national forests, state parks, and farm woods; inhabited soils, collected from residential lawns, city parks, lawns adjacent to public buildings, institutions and industrial plants, excavated soil, bird nesting areas, an animal burrow, and a rural camping location; cultivated soils, which included wheat, oats, maize and beet fields, tomato, strawberry and melon patches, greenhouse soils, an apple orchard and a compost heap; fallow fields, ploughed fields, and irrigated farm soil; polluted soils, which were samples from the various soil classes which were known to be in contact with recent contamination.

Sampling procedure and examination of samples. The samples were collected by scraping away approximately one inch of surface soil and filling a sterile 1 oz ointment tin with the soil under the denuded area. Samples of 10 g of each soil suspended in 90 ml of buffered sterile water were examined by the confirmed multiple tube procedure for the coli-aerogenes group, the completed test as described in *Standard Methods for the Examination of Water and Wastewater* (Methods, 1960), and the faecal coli-aerogenes tests, all of which were based on 5 tubes at each of dilutions and gave results as most probable numbers (MPN). The faecal test was accomplished by confirming presumptive positive tubes in EC broth at 44·5°±0·5°. This elevated temperature test has been shown by Geldreich *et al.* (1962) to have a 96·3% positive correlation with coli-aerogenes strains found in warm blooded animals. The membrane filter technique was used to effect a preliminary isolation of the organisms. A minimum of 25–50 isolated colonies with a typical sheen were transferred to phenol red lactose broth for fermentation tests, verified in brilliant green–lactose–bile broth, and then streaked on eosin–methylene blue agar (EMB) to ensure cultural purity. The strains were then ready for simultaneous inoculations into boric acid–lactose broth (BALB) and EC broth to test for fermentation at 43° and 45°, respectively, and also into the necessary media for identification and classification by the IMViC tests. The elevated temperature procedures were those described by Vaughn, Levine & Smith (1951) for BALB and a modification of the Hajna & Perry (1943) EC test. The IMViC procedures used were those described in Methods (1960).

RESULTS

The results of the 5-tube confirmed, completed and elevated temperature (faecal coli-aerogenes bacteria) tests for each of the soil classes are summarized in Table 1. Many samples from the arid, subterranean, submerged, pasture and shore-line soil classes were found to contain <1·8 coli-aerogenes bacteria/g at the 25th percentile point as determined by the completed test. These organisms were not detected in any of the seven 'arid' samples. The upper quartile values, respectively, for the completed

Table 1. *The percentile distribution of MPN values/g of sample for* 251 *soils*

Soil class	No. of samples	Counts (MPN/g) in quartile grades according to								
		Confirmed test			Completed test			Faecal test		
		25%	50%	75%	25%	50%	75%	25%	50%	75%
Arid	7	<1·8	<1·8	<1·8	<1·8	<1·8	<1·8	<1·8	<1·8	<1·8
Subterranean	9	<1·8	2·0	230	<1·8	<1·8	12	<1·8	<1·8	<1·8
Submerged	14	<1·8	4·5	13	<1·8	2·0	13	<1·8	<1·8	13
Pasture	23	<1·8	150	2,500	<1·8	20	170	<1·8	3·0	17
Shore line	58	<1·8	200	3,300	<1·8	75	430	<1·8	<1·8	49
Woodland	56	6·8	200	14,000	1·8	17	2,900	<1·8	<1·8	11
Inhabited	21	33	2,600	33,000	8·3	84	2,200	<1·8	<1·8	18
Cultivated	35	170	3,000	13,000	8·1	150	1,800	<1·8	<1·8	11
Polluted	28	23,000	64,000	330,000	9,500	48,000	220,000	33,000	11,000	49,000

test were 12 and 13 coli-aerogenes bacteria/g for subterranean and submerged samples, and 170 and 430 for pastures and shore-line soils. The 25% and 75% quartile ranges for woodland, inhabited and cultivated soils were 1·8–2,900; 8·3–2,200; and 8·1–1,800/g, respectively. The density for polluted samples ranged from 9,500–220,000/g. The faecal coli-aerogenes content of all eight unpolluted soil classes was very low, with the majority of samples indeterminate (i.e. <1·8/g). The number of faecal coli-aerogenes organisms for polluted samples ranged from 3,300–49,000/g for the 25 to 75 percentile points.

The results of repeated sampling from four soils during irregular intervals over an 8–10 month period are reported in Table 2. The results of the completed tests for samples from the oat field varied from 14–4,600/g and those for the mountain meadow from 6·1–17,000/g. A variation of <1·8–250/g occurred from Flynn Creek timberland. The results from Needle Creek timberland were in the range of <1·8–26/g. The faecal coli-aerogenes results were consistently low and 15 of the 26 MPN values were <1·8. The highest faecal coli-aerogenes density of 110 was found in one oatfield sample and the next two highest were 45 and 33.

Table 2. *The variation in counts of coli-aerogenes bacteria obtained on repeat samples of soil from four sources*

Source of sample	Interval between samples (days)	Counts (MPN/g) obtained in		
		Confirmed test	Completed test	Faecal test
Oat field	0	4,600	4,600	110
	16	700	220	11
	26	170,000	170	45
	17	2,200	17	<1·8
	144	7,900	14	<1·8
Mountain meadow	0	79	13	7·8
	15	700	64	1·8
	51	15,000	6·1	<1·8
	61	7,900	1,400	11
	144	17,000	17,000	<1·8
Timberland (Flynn Creek)	0	11	11	11
	15	1·8	<1·8	<1·8
	14	49	49	<1·8
	37	220	13	<1·8
	21	3,500	31	<1·8
	33	84	46	33
	17	13,000	250	4·5
	144	1·8	<1·8	<1·8
Timberland (Needle Branch)	0	7·8	7·8	7·8
	15	<1·8	<1·8	<1·8
	14	<1·8	<1·8	<1·8
	37	7·8	7·8	<1·8
	21	<1·8	<1·8	<1·8
	33	70	26	<1·8
	17	11,000	7·3	<1·8
	144	6·8	4·5	2·0

During this investigation 3,013 strains of coli-aerogenes organisms were examined. This represented 20–100 strains from each of 54 undisturbed soils and 13 polluted soils. The predominant IMViC types in the undisturbed soils were – + – + (48·1%)

and − − + + (18·8%). Seven other types occurred with a frequency greater than 2%. With reference to the polluted soils, 98·9% of the total coli-aerogenes organisms were represented by 4 types (+ + − −, − + − +, + + − + and − − + +). No single type in the occasionally occurring groups from either undisturbed or polluted soils constituted more than 1% of the coli-aerogenes content.

DISCUSSION

The results of the confirmed EC elevated temperature procedure indicate that faecal coli-aerogenes bacteria are usually absent, or present only in comparatively small numbers, in undisturbed soils. There was, however, a sharp increase in the numbers of these types from soils known to be polluted. This is in agreement with a study by Allen, Grindley & Brooks (1953) of bottom deposits from lakes and estuaries. Their elevated temperature test confirmed all positive MacConkey's broth presumptive tubes (37°) in the same medium at 44°. Randall (1956) modified this technique by confirming in MacConkey's agar roll tubes at 44°. He concluded that the density of *E. coli* in soil was directly proportional to the degree of probable pollution. Thus, three different elevated temperature procedures have supported the same conclusion about the occurrence of faecal coli-aerogenes bacteria in soil; i.e., that the more remote an area is from human and animal pollution, the less likely are faecal types to be found.

Intermediates have been reported frequently to constitute a considerable proportion of the coli-aerogenes bacteria in soils. In this study, 48·1% of 2,348 strains from 54 undisturbed soils were of an intermediate type giving the IMViC reactions − + − + (Table 3). This type also comprised 51·6% of 289 strains recovered from a protected

Table 3. *The occurrence of different IMViC types of coli-aerogenes bacteria in 67 samples of soil*

IMViC results	Strains isolated from soils			
	Undisturbed*		Polluted†	
	No.	%	No.	%
+ + − −	131	5·6	536	80·6
− − + +	443	18·8	13	2·0
− + − −	78	3·3	1	0·2
+ + + −	7	0·3	0	0
− + − +	1,131	48·1	87	13·0
+ + − +	87	3·7	22	3·3
− + + +	181	7·7	5	0·7
+ + + +	159	6·8	0	0
+ − + +	67	2·9	0	0
− − − +	4	0·2	1	0·2
− + + −	1	‡	0	0
− − + −	53	2·3	0	0
+ − + −	6	0·3	0	0

* In all 54 soils were examined and 2,348 cultures isolated and studied; 216 (9·2%) and 200 (8·5%) of the cultures were positive in EC and BALB media, respectively.

† In all 13 soils were examined and 665 cultures isolated and studied; 551 (82·9%) and 545 (82·0%) of the cultures were positive in EC and BALB media, respectively.

‡ Insufficient cultures studied.

raw water supply for a large western city. Since the entire area was enclosed by fence, birds or small rodents were probably the sources of the 1·04% of faecal coli-aerogenes bacteria found. Thus, it is likely that the origin of the high percentage of IMViC type – + – + in this raw water supply was the soil along the watershed. There were 75·6% of intermediate strains in the 2,348 cultures isolated from undisturbed soils, whereas only 17·4% of the 665 strains from polluted soils were intermediate types. In the protected raw water supply of the western city cited, 93·1% of the 289 strains were intermediate in type, giving IMViC results between + + – – and – – + +.

These data provide strong circumstantial evidence that the IMViC – + – + type of coli-aerogenes organism is predominantly a soil type. This conclusion is based not only upon the results of the present study, in which 48·1% of such a type were present, but also on the very low occurrence of IMViC type – + – + (0·6%) in faecal samples, as described by Geldreich *et al.* (1961), and the occurrence of this type in certain mountain streams studied at various times in the past.

Since soil abounds with many anaerobic and aerobic sporeforming bacteria, and symbiosis is common, it was not unusual to observe large discrepancies in confirmed and completed tests. The largest deviation occurred in a soil collected from a creek bank, for which the confirmed MPN value was $1{\cdot}3\times10^6$/g, whereas the completed MPN result was only 270/g. It thus appears that the combination of the chemical agents, lauryl sulphate in the presumptive broth and brilliant green plus bile salts in the confirmatory medium, was ineffective in suppressing the growth of some bacteria which were not coli-aerogenes organisms. Because of this frequency of false positive reactions, the completed test must be used in preference to the confirmed procedure in the examination of soils.

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REFERENCES

Allen, L. A., Grindley, J. & Brooks, E. (1953). Some chemical and bacterial characteristics of bottom deposits from lakes and estuaries. *J. Hyg., Camb.*, **51**, 185.

Bardsley, D. A. (1934). The distribution and sanitary significance of *B. coli*, *B. lactis aerogenes* and the intermediate types of coliform bacilli in water, soil, faeces and ice cream. *J. Hyg., Camb.*, **34**, 38.

Chen, C. C. & Rettger, L. F. (1920). A correlation study of the colon-aerogenes group of bacteria with special reference to the organisms occurring in the soil. *J. Bact.* **5**, 253.

Clark, H. F., Geldreich, E. E., Kabler, P. W., Bordner, R. H. & Huff, C. B. (1957). The coliform group. I. The boric acid lactose broth reaction of coliform IMViC types. *Appl. Microbiol.* **5**, 396.

Frank, N. & Skinner, C. E. (1941). Coli-aerogenes bacteria in soil. *J. Bact.* **42**, 143.

Geldreich, E. E., Clark, H. F., Kabler, P. W., Huff, C. B. & Bordner, R. H. (1958). The coliform group. II. Reactions in EC medium at 45°C. *Appl. Microbiol.* **6**, 347.

Geldreich, E. E., Bordner, R. H., Huff, C. B., Clark, H. F. & Kabler, P. W. (1962). Type distribution of coliform bacteria in the feces of warm-blooded animals. *J. Wat. Polut. Contr. Fed.* **34**, (in press).

GRIFFIN, A. M. & STUART, C. A. (1940). An ecological study of the coliform bacteria. *J. Bact.* **40**, 83.

HAJNA, A. A. & PERRY, C. A. (1943). Comparative study of presumptive and confirmatory media for bacteria of the coliform group and for fecal streptococci. *Amer. J. Publ. Hlth* **33**, 550.

JOHNSON, B. R. & LEVINE, M. (1917). Characteristics of coli-like microorganisms from the soil. *J. Bact.* **2**, 379.

KOSER, S. A. (1926). The coli-aerogenes group in soil. *J. Amer. Wat. Wks Ass.* **15**, 641.

METHODS (1960). *Standard Methods for the Examination of Water and Wastewater*, 11th ed. New York, N.Y.: American Public Health Association, Inc.

PARR, L. W. (1939). Coliform bacteria. *Bact. Rev.* **3**, 1.

RAGHAVACHARI, T. N. S. (1926). The application of Koser's citrate utilization tests to sanitary water analysis in India. *Ind. J. med. Res.* **14**, 47.

RANDALL, J. S. (1956). The sanitary significance of coliform bacilli in soil. *J. Hyg., Camb.*, **54**, 365.

TAYLOR, C. B. (1951). Coli-aerogenes bacteria in soils. *J. Hyg., Camb.*, **49**, 162.

VAUGHN, R. H., LEVINE, M. & SMITH, H. A. (1951). A buffered boric acid lactose medium for enrichment and presumptive identification of *Escherichia coli*. *Food Res.* **16**, 10.

(*Received* 30 *September*, 1961)

Barnes, E. M. & Goldberg, H. S. (1962). *J. appl. Bact.* **25** (1), 94–106.

THE ISOLATION OF ANAEROBIC GRAM-NEGATIVE BACTERIA FROM POULTRY REARED WITH AND WITHOUT ANTIBIOTIC SUPPLEMENTS

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SUMMARY: A number of methods advocated for the isolation of 'bacteroides' have been tested. By using an ethyl violet-azide agar medium under strictly anaerobic conditions, as many as 10^8 g of a group of obligately anaerobic Gram-negative bacteria were detected in the caeca of chickens. Freshly isolated organisms from chlortetracycline fed chickens showed a slightly greater resistance to chlortetracycline than those isolated from the control birds, but there was little difference after further subculture.

It is well known that many of the nonsporing obligate anaerobes present in the alimentary tract of animals cannot be isolated unless special procedures are used. This study was undertaken to see whether there is in the caeca of poultry a large population of organisms which was overlooked in an earlier study of the chicken flora by Barnes & Shrimpton (1957). At the same time it was decided to compare in this respect chickens fed on control diets with those fed on diets containing antibiotic supplements, since it was previously shown that coli-aerogenes organisms, salmonellae and faecal streptococci isolated from chickens reared on chlortetracycline-supplemented diets were much more resistant than those isolated from control birds (Barnes, 1958; Gordon, Garside & Tucker, 1959; Garside, Gordon & Tucker, 1960).

Considerable confusion exists over the identification and classification of the Gram-negative anaerobes. Early workers differentiated between the genera *Bacteroides* and *Fusiformis* morphologically and ecologically, *Bacteroides* being associated with the intestinal flora (Weiss & Rettger, 1937; Eggerth & Gagnon, 1933), whereas *Fusiformis* strains were isolated from the mouth (Slanetz & Rettger, 1933; Hine & Berry, 1937). The seventh edition of Bergey's *Manual* (Breed, Murray & Smith, 1957) recognized 5 genera which are still differentiated only on morphological characteristics. Beerens (1953–1954, 1957) has made a special study of the genera *Ristella*, *Sphaerophorus* and *Fusiformis*, which he divided mainly on the basis of a number of biochemical tests as well as the behaviour of the organisms towards various inhibitors.

Difficulties in the cultivation and preservation of *Bacteroides* spp. were described by Eggerth & Gagnon (1933), who confirmed the necessity for including reducing substances such as cysteine in the medium. Lewis, Bedell & Rettger (1940) also showed that growth was greatly improved by incubation in hydrogen containing 10% of CO_2 rather than in hydrogen alone. The fusiforms require similar conditions of growth; in addition, gentian violet or ethyl violet has frequently been used in their isolation from the mouth because of the need to suppress the numerous lactobacilli also present (Slanetz & Rettger, 1933). More recently the use of antibiotics has been suggested for selecting both *Bacteroides* spp. and fusiforms. Baird-Parker (1955) showed that *Fusobacterium* spp. would grow in the presence of 15 p/m of neomycin sulphate, 20 p/m of streptomycin or 0·001 mg of bacitracin/ml. Finegold & Hewitt (1955–56) suggested that neomycin might be useful in a selective medium for *Bacteroides* spp. Stevens & Harrison (1958) found that the *Bacteroides* spp. they tested were resistant to penicillin and dihydrostreptomycin, but sensitive to chloramphenicol and chlortetracycline. Beerens (1953–54) stated that the genus *Ristella* was generally inhibited either by gentian violet or by brilliant green but was stimulated by bile. For these organisms he used a selective medium containing 20% of bile and sodium azide ($1:10^4$), whereas for *Sphaerophorus* spp. and a number of *Fusiformis* strains he used brilliant green ($1:14\times10^4$) and sodium azide ($1:10^4$) (Beerens, 1957).

As it was not known which of the genera would be present in the intestines of poultry, all the procedures suggested for the isolation of Gram-negative obligate anaerobes were employed. However, the selectivity of a medium depends largely upon the other bacteria present and it was soon found that none of the selective media or techniques already published could be used without modification for isolating these organisms from the caeca of young chickens. Much of this paper is therefore concerned with the isolation procedures. The term 'bacteroides' is used to denote any of the obligately anaerobic, Gram-negative, nonsporing bacteria which would be included in the family Bacteroidaceae.

METHODS

Rearing and feeding of birds

Birds from two different sources were used in these experiments. First, birds of 6–9 months of age were kept at the Low Temperature Research Station and fed on a standard diet (Beattie & Shrimpton, 1958) without any antibiotic supplements. Second, three groups of birds were reared at the Houghton Poultry Research Station under identical conditions on a standard broiler diet, one group being fed on the control ration without antibiotics, a second having the control ration with 25 μg of chlortetracycline/g throughout life and the third group having 100 μg of chlortetracycline/g throughout life.

Caecal samples

Caecal samples from older live birds were obtained as caecal droppings. Care was taken that these droppings were left in the cage for a very short time only (usually less than 15 min) before weighing and testing. Young birds, reared at the Houghton Poultry Research Station, were killed, the abdominal cavity opened immediately and the caecal contents squeezed into a weighed bottle containing diluent. Usually birds (killed at intervals from 17 days to 13 weeks of age) from each group were tested.

In every case the caecal material was emulsified in a previously weighed 1 oz bottle containing 19 ml of Reinforced Clostridial Medium (RCM, Oxoid), which had been heated to remove oxygen and cooled. Glass beads were frequently used in the diluent bottle to help mix the sample. The cap of the bottle was screwed down tightly and the bottle gently rotated to disperse the sample without unnecessary aeration.

Culture media

In the experiments described below a number of different media were used. The composition of the media (as % w/v unless otherwise stated) and the method of use were as follows:

Liquid reinforced clostridial medium (*RCM*, *Oxoid*). This broth was used throughout in 19 ml lots in 1 oz bottles as a diluent and also as a growth medium. It was always heated in a boiling water bath for about 20 min and then cooled shortly before use. After this treatment concentrated aqueous solutions of various inhibitors were added to give final concentrations as follows: neomycin sulphate (715 μg/mg), 15, 45 and 500 μg/ml; penicillin, 1, 5, 10, 20, 40 and 100 units/ml; polymyxin B sulphate (7,000 units/mg), 50 μg/ml; streptomycin sulphate (740 μg/mg), 50 μg/ml; sodium azide, $1:10^4$, $1:2\times10^4$ and $1:4\times10^4$; Bacto-oxgall, 10 and 20% (v/v); ethyl violet (having an 87% dye content, G. T. Gurr), $1:2\times10^4$ and $1:5\times10^4$.

Reinforced clostridial agar (*RCM agar*, *Oxoid*). This was used alone and also containing various concentrations of inhibitors which were added immediately before the plates were poured. The inhibitors and the concentrations used were: chlortetracycline hydrochloride, 0·1, 1·0 and 10 μg/ml; sodium azide, $1:10^4$, $1:2\times10^4$ and $1:4\times10^4$; and ethyl violet, $1:2\times10^4$ and $1:5\times10^4$.

Ethyl violet-azide agar (*EVA agar*). This was RCM agar containing ethyl violet ($1:2\times10^4$) and sodium azide ($1:2\times10^4$). Ethyl violet (5 ml/l. of a 1% aqueous solution) was added to dissolved RCM agar. The medium was sterilized in 100 ml lots and 0·5 ml of a 1% solution of sodium azide added immediately before pouring the plates.

Basal medium of Beerens. The basal medium of Beerens (1953–1954) was slightly modified and had the following composition: peptone (Evans), 1·0; sodium chloride, 0·5; Lab-Lemco, 0·3; yeast extract (Oxoid), 0·5; cysteine hydrochloride, 0·04; agar (New Zealand), 0·06; pH 7·4. It was sterilized at 15 lb/in^2 for 15 min in 19 ml lots in 1 oz bottles and used in the identification tests for the anaerobic Gram-negative bacteria following the methods of Beerens (1953–1954).

Beerens basal medium with glucose and phosphate (*BGP*). To the above basal medium were added glucose (0·1%) and Na_2HPO_4 (0·4%). This medium was used for the maintenance of stock cultures for limited periods.

BGP plus gelatin. Gelatin (1·5%) was incorporated in the above BGP medium. This was also used for stock cultures.

Sodium formaldehyde–sulphoxalate was sometimes added to the three media containing Beerens basal medium to lower the redox potential (Riemann, 1957). This was done by adding a 3% aqueous solution of the compound (sterilized by filtration) to the medium, immediately before inoculating with the test organism, to give a final concentration of 0·03%.

Viable counts

All the dilutions were prepared in a 20-fold series (1/20, 1/400, 1/8,000, 1/16,000, etc.) by taking 1 ml of the emulsified caecal material and mixing with a further 19 ml of RCM broth, and so on in series. From these dilutions aliquots were removed for plate counts or into RCM broth containing the different inhibitors. The remainders of the dilution broths were always incubated at 37° for several days.

Gram-negative obligate anaerobes (*'bacteroides'*). The final method used was as follows: a volume of 0·05 ml of each dilution was rapidly spread over the surface of half a freshly prepared plate of agar. Generally, triplicate plates were prepared for each pair of dilutions, the number of plates being limited by the time available and by the capacity of the anaerobic jar used. It was found essential that only one sample should be handled at a time and that the total time of exposure of the organisms on the surface of the plates should be less than 10 min. The plates were incubated anaerobically in hydrogen containing 10% of CO_2. It was also found advisable to remove the last traces of oxygen from the anaerobic jar by heating the palladium catalyst without delay. The plates were incubated for 2–3 days at 37°. Colonies of the 'bacteroides' were very large (about 3–4 mm in diameter), generally smooth and deep purple in colour.

Coli-aerogenes bacteria. These were counted by spreading 0·025 ml of the above dilutions over one quarter of the surface of plates of MacConkey's agar (Oxoid no. 3).

Lactobacilli. The medium of Rogosa as modified by Mabbitt & Zielinska (1956) was used. The plates were incubated for 2 days at 37° in an atmosphere of 90% of hydrogen and 10% of CO_2.

Group D streptococci. The organisms were isolated on thallous acetate–tetrazolium agar (pH 6·0) after incubation for 24 hr at 37° (Barnes, 1956).

Purification and preservation of the 'bacteroides'

Preparation of stock cultures. As soon as the incubated EVA plates had been removed from the anaerobic jar, typical colonies of 'bacteroides' were streaked directly on freshly poured RCM agar plates which were again incubated without delay in an atmosphere of the same composition as before.

After a further incubation for 2 days at 37° the typical 'bacteroides' colonies were picked from the RCM agar plates into RCM broth. From here working stock cultures were prepared in BGP broth or BGP plus gelatin. The caps of the tubes were sealed over with paraffin wax and the cultures stored at 1°, under which conditions they remained viable for about one month. For permanent storage the cultures were freeze dried.

Freeze drying of 'bacteroides'. The organisms were grown for 2 days at 37° in RCM broth, then centrifuged, and all but 1 ml of the supernatant poured off. To the residue was added 1 ml of a 16% (w/v) solution of glucose which had previously been heated in boiling water for about 10 min to remove oxygen. The broth plus glucose containing the centrifuged organisms was then distributed rapidly into ampoules and dried in a centrifugal freeze drier (Edwards High Vacuum Ltd., Crawley, Sussex). The dried cultures were stored at 1° and resuscitated in RCM broth when required.

Characterization of Gram-negative anaerobes

The organisms were examined for characteristic morphology by phase contrast microscopy and after staining by Gram's method. Biochemical tests (carbohydrate fermentation, H_2S production, gelatin liquefaction, etc.) were carried out following the techniques and classification described by Beerens (1953–1954). Beerens basal medium containing 0·25% glucose was used to determine the final pH and the organic acids produced.

The following strains were used for comparative purposes: *Sphaerophorus funduliformis, Sphaer. freundii, Sphaer. necrophorus, Diplobacillus* 22 and 25, *Ristella convexa* R242, R250 and R265, *Ristella incommunis* R294 and R308 (all kindly supplied by Dr. H. Beerens of the Institut Pasteur, Lille), *Bacteroides* ATTC 7155 and 9343, *Bacteroides tumidis* ATCC 8496, *Bacteroides fragilis* NCTC 9338 and *Bacteroides necrophorus* NCTC 7155.

Determination of antibiotic sensitivities

Freshly isolated strains of coli-aerogenes bacteria. The MacConkey's no. 3 agar plates used for the enumeration of the coli-aerogenes strains in the chicken caeca were replicated by the technique of Lederberg & Lederberg (1952) on to Bacto heart infusion agar containing 1 and 10 μg of chlortetracycline/ml. At the same time they were also replicated on to blood agar to determine any difference in haemolytic properties.

Freshly isolated strains of 'bacteroides'. As soon as the EVA plates used for counting the 'bacteroides' in the chickens had been removed from the anaerobic jar, the plates showing discrete colonies of 'bacteroides' were immediately replicated on two RCM agar plates and on RCM agar plates containing 1 and 10 μg of chlortetracycline/ml. One of the RCM agar plates was incubated aerobically at 37° to confirm that the colonies were in fact obligate anaerobes and the other three plates were incubated immediately in an atmosphere of H_2 containing 10% of CO_2 for two days at 37°.

Purified strains of 'bacteroides'. Pure cultures of the test organisms were grown in RCM broth and then were streaked by means of a loop radially on RCM agar plates, 7–8 cultures being inoculated on to each plate. The plates were incubated anaerobically for 2 days at 37° and then replicated as before on RCM agar and RCM agar containing 0·1, 1 and 10 μg of chlortetracycline/ml. These plates were incubated anaerobically for 3 days at 37°.

RESULTS

The isolation of 'bacteroides' from chicken caeca

It was originally intended to determine the total number of 'bacteroides' in the caeca of chickens at intervals from 17 days to 13 weeks. Their isolation was however complicated by the presence of large numbers of lactobacilli, coli-aerogenes bacteria and faecal streptococci. The caeca of chickens aged 17 days were found to contain lactobacilli in numbers from $<2\times10^7$ to $1{\cdot}2\times10^{10}$, coli-aerogenes bacteria from $2{\cdot}2\times10^8$ to $6{\cdot}4\times10^9$ and faecal streptococci from $2{\cdot}8\times10^8$ to $1{\cdot}6\times10^9$/g. In older birds of 6–9 months of age coli-aerogenes bacteria were present in much smaller numbers and the streptococci and lactobacilli were not determined. Thus the 'bacteroides' had to be separated from both the streptococci and lactobacilli, as well as from the coli-aerogenes organisms; the coli-aerogenes separation was the most difficult.

The use of inhibitors in RCM broth. This was used throughout as the basal medium because the chicken 'bacteroides' grew well in it. Media were generally tested first with the caecal droppings from the older birds and then, if they showed any promise, the same media were tested with caecal samples from young chickens. After preparing the sample and diluting as described, 1 ml from each dilution was added to RCM broth containing the selective agent. After incubation for 1 day at 37° the broths were examined microscopically for the presence of Gram-negative bacteria. It was not easy to differentiate the 'bacteroides' from the coli-aerogenes bacteria, although the former were generally much larger, of a different shape and stained only weakly. If the 'bacteroides' were thought to be present, attempts were then made to isolate them. The results are shown in Table 1.

In older birds, where the coli-aerogenes bacteria were outnumbered by the 'bacteroides', the latter could be isolated by using either penicillin (1 or 10 units) or ethyl violet ($1:2\times10^4$), both of which suppressed the lactobacilli and streptococci. In the young birds, where the coli-aerogenes bacteria apparently outnumbered the 'bacteroides', none of the inhibitors tested inhibited the coli-aerogenes while allowing the 'bacteroides' to grow. In every case where 'bacteroides' were observed microscopically to be growing together with coli-aerogenes bacteria, it proved impossible to isolate them. It is possible that the acidity produced by the coli-aerogenes bacteria in RCM broth destroyed the 'bacteroides'. For whatever reason, it was evident that none of the media could be used for presumptive 'bacteroides' counts, so solid media were then tested for isolating these organisms.

The effect of ethyl violet and sodium azide in RCM agar. It was first established that the few 'bacteroides' isolated in the above tests could be recovered quantitatively when grown on the surface of RCM agar under hydrogen plus 10% of CO_2 using the precautions described above. Ethyl violet at a concentration of $1:2\times10^4$ or $1:5\times10^4$ was then incorporated in RCM agar and sodium azide at a concentration of $1:10^4$, $1:2\times10^4$ or $1:4\times10^4$ added immediately before pouring the plates. The plates were streaked with actively growing cultures of the chicken 'bacteroides' and also with known strains of related Gram-negative organisms. The results are summarized in Table 2. It was evident that the least inhibitory medium was the one containing $1:5\times10^4$ ethyl violet plus $1:4\times10^4$ sodium azide, but unfortunately the coli-aerogenes bacteria also grew well. Sodium azide at $1:10^4$ inhibited both the coli-aerogenes bacteria and the 'bacteroides'. It seemed that a concentration of $1:2\times10^4$ of sodium azide together with $1:2\times10^4$ or $1:5\times10^4$ of ethyl violet would give the best results. Trials were then carried out using the caecal contents from young chickens, where the coli-aerogenes population would be highest. It was found that a considerable number of lactobacilli and streptococci grew as well as the 'bacteroides' in the presence of the $1:5\times10^4$ ethyl violet; the coli-aerogenes organisms formed a background haze only, as they were considerably inhibited by the sodium azide. When $1:2\times10^4$ of ethyl violet was used the lactobacilli and streptococci were completely suppressed, the 'bacteroides' appeared as large purple colonies and the coli-aerogenes

Table 1. *The selective action of various inhibitors in RCM broth on the caecal flora of chickens*

Age of bird (weeks)	Inhibitor, and concn. used							Log_{10} highest dilution showing growth in RCM broth with inhibitors		Organisms* present in broth with inhibitor
	Neomycin (µg/ml)	Penicillin (units/ml)	Polymyxin (µg/ml)	Bile (%)	Sodium azide†	Ethyl violet†	Strepto-mycin (µg/ml)	Abs-ent	Pres-ent	
26	15							9	9	G+ rods and some G− rods
	45							9	9	
	500							9	6	G+ rods
2½		1						9–10	7	G− rods, A
26		1						9	6	G− rods, B
2½		10						9–10	7–9	G− rods, A
6		10						9	7	
6½		10						9–10	6–7	
34		10						9	3	Mainly G− rods, A and B
6		20						9	4	G− rods, A
6		40						9	1	
6		100						9	<1	
26			50					9	9	G+ and G− rods
26		10	50					9	9	
26				10	10^4			9	7	G+ and G− rods and cocci
34		10		20	10^4			9	2	G+ rods
2½		10			10^4			9	<2	
34		10			10^4			9	3	G+ rods
2½		5			10^4			9	<2	
2½		2·5			10^4			9	<2	
39					10^4	2×10^4		9	2	G+ rods and cocci
39					10^4	5×10^4		9	2	
2½		10			2×10^4			9	2–3	G+ rods
2½		10			4×10^4			9	2–3	
6½					4×10^4	2×10^4		9	4	G+ and G− rods, A and B
39					4×10^4	2×10^4		9	4	
2½						2×10^4		9	6	G− rods, mainly A
26						2×10^4		9	5	G− rods, B
39						2×10^4		9	4	G− rods, A and some G+ rods
39		10				2×10^4		9	3	G− rods, A and B
39						5×10^4		9	4	G+ rods and cocci and some G− rods
26						5×10^4	50	9	6	G+ rods and cocci
39		10				5×10^4		9	4	G− rods, mainly A
6½					4×10^4	5×10^4		9–10	5–6	Mainly G− rods
39					4×10^4	5×10^4		9	4	G+ and G− rods and cocci

* G+, Gram-positive; G−, Gram-negative; A. coli-aerogenes bacteria; B, 'bacteroides'.
† Vol. (ml) containing 1 g.

organisms again formed a background haze. This medium was therefore chosen for the isolation of the 'bacteroides' from the chickens, although it was appreciated that only a minimal count could be obtained because some of the standard strains tested failed to grow (Table 2). Following a suggestion by Mossel (1959) that blood improved growth and recovery, 5 % of horse blood was added to the medium in one series of experiments. Although the recovery of 'bacteroides' was slightly improved the medium was also less inhibitory for the lactobacilli, making isolation and counting more difficult.

Table 2. *The effect of ethyl violet and sodium azide in RCM agar on the growth of 'bacteroides' and coli-aerogenes bacteria*

Identity of cultures	Growth† on control RCM agar	Growth† on RCM agar with ethyl violet at $1:5\times10^4$ (I) or $1:2\times10^4$ (II) and sodium azide at					
		$1:4\times10^4$		$1:2\times10^4$		$1:10^4$	
		I	II	I	II	I	II
46/1*	++++	++++††	++	nt	nt	+	–
	++++	+++	++	+++	++	nt	nt
46/2*	++++	+++	+	nt	nt	+	±
48/33*	++++	++++	+++	nt	nt	++++	++
Coli-aerogenes (1)	+++§	++	++	nt	nt	±	±
Coli-aerogenes (2)	+++§	+++	+++	nt	nt	±	–
Coli-aerogenes (3)	++++	++++§§	++++§§	+	+	nt	nt
Sphaerophorus funduliformis	++++	++++	++++	nt	nt	++++	++++
Sphaer. necrophorus	++++	++++	++++	nt	nt	++++	++++
Diplobacillus 22	++++	–	–	nt	nt	–	–
Bacteroides sp. (ATCC 7155)	+++	–	–	nt	nt	–	–
Ristella variabilis (R264)	++	nt	nt	nt	±	–	–
Rist. convexa‡ *Rist. incommunis*‡‡	+§	nt	nt	nt	nt	nt	nt

* Cultures isolated from chickens. †Growth: ++++, good, large number of colonies; +++, moderate; ++, fair; +, a few isolated colonies; ±, trace; –, no growth.

†† Colonies smaller than control. § Colonies small. §§ Colonies very small.

‡ Three strains tested. ‡‡ Two strains tested. nt, not tested.

Recovery of 'bacteroides' on EVA agar. Three cultures of 'bacteroides' were chosen: one was isolated from chickens using an EVA agar plate, one from EVA agar plus horse blood and one which was inhibited by the EVA agar but which had grown when replicated on RCM agar. The cultures were all grown in RCM broth and then diluted as described above and plated on RCM agar, EVA agar and EVA agar plus blood. The results, shown in Table 3, confirm that probably only a small proportion of the 'bacteroides' were recovered on this medium. They also show that the addition of blood to the EVA agar improved the medium only slightly.

Table 3. *The influence of the plating medium on the recovery of three 'bacteroides' strains isolated on different agar media and grown in RCM broth*

Culture no.	Medium* used for isolation	Viable count (/ml of RCM broth) on		
		RCM agar* (control)	EVA agar* +5% blood	EVA agar*
61/42	EVA agar	$7\cdot5\times10^6$	$3\cdot5\times10^6$	$1\cdot2\times10^6$
61/28	EVA agar +5% blood	$6\cdot2\times10^6$	9×10^3	†
61/62	RCM agar	$4\cdot8\times10^6$	7×10^3	†

* Media: EVA agar, ethyl violet–azide agar; RCM agar, reinforced clostridial medium agar.

† No discrete colonies obtained.

Purification and identification of organisms from EVA agar plates

For a considerable time great difficulty was encountered in purifying the large colonies of 'bacteroides'. When a colony was subcultured into RCM broth the small number of coli-aerogenes organisms forming the background haze on the plates was carried over and grew, and no 'bacteroides' were isolated. The use of broth containing azide did not help, as the concentrations inhibitory for the coli-aerogenes also inhibited the 'bacteroides'. The procedure finally adopted was that described above for the preparation of stock cultures and proved most satisfactory, provided there was no delay when plates were removed from the anaerobic jar.

Using these methods over 100 strains were isolated from both the older birds and the groups of young birds. When grown on RCM agar they generally formed colonies 3–5 mm in diameter which were either smooth or, more frequently, tended to have a fimbriate edge and a matt appearance. The bacteria themselves consisted of extremely large rods with rounded ends. When grown in RCM broth they varied from 4·5–12 μ in length and about 1·5–2·0 μ in width. They were very difficult to stain by Gram's method. Examined under phase contrast they showed granules and had a banded appearance. Spindle forms were observed in colonies on some of the selective media. They fermented a wide range of carbohydrates including glucose, lactose, maltose, xylose, mannitol, raffinose, arabinose, galactose, cellobiose and fructose, with a final pH in the culture fluid of 4·8–5·4. These organisms differed morphologically from the *Ristella* strains of Beerens and also in their behaviour both on RCM agar and on the selective media (Table 2); moreover they were not stimulated by bile (Beerens & Castle, 1960) nor inhibited by brilliant green. It appears

Table 4. *The influence of chlortetracycline (CTC) in the diet of birds on the numbers of 'bacteroides' and coli-aerogenes bacteria in their caeca*

Expt. no.	Age of birds (weeks)	Organisms isolated	Nos./g of caecal matter from birds* on		
			Control diet	Feed + CTC at 25 μg/g	Feed + CTC at 100 μg/g
1	2½	'Bacteroides'†	8×10^4	2×10^3	1×10^5
			2×10^7	4×10^4	nt
		Coli-aerogenes	$4{\cdot}4\times10^8$	$2{\cdot}1\times10^7$	5×10^8
			$4{\cdot}3\times10^8$	1×10^8	$4{\cdot}9\times10^7$
	7	'Bacteroides'†	1×10^4	$1{\cdot}5\times10^5$	6×10^4
			2×10^5	1×10^4	nf
		Coli-aerogenes	$<3{\cdot}2\times10^5$	$1{\cdot}2\times10^6$	$2{\cdot}9\times10^7$
			$4{\cdot}9\times10^6$	$1{\cdot}6\times10^7$	8×10^5
	8	'Bacteroides'†	8×10^5	$2{\cdot}5\times10^3$	5×10^3
			6×10^3	2×10^4	4×10^4
		Coli-aerogenes	$6{\cdot}8\times10^6$	$3{\cdot}4\times10^6$	$2{\cdot}2\times10^7$
			$3{\cdot}2\times10^7$	$6{\cdot}4\times10^7$	$1{\cdot}5\times10^7$
	13	'Bacteroides'†		6×10^6	2×10^3
				4×10^5	1×10^3
		Coli-aerogenes		$8{\cdot}3\times10^7$	$2{\cdot}8\times10^6$
				$4{\cdot}1\times10^6$	$3{\cdot}2\times10^6$
2	12	'Bacteroides'	$6{\cdot}1\times10^7$		$2{\cdot}3\times10^7$
			$3{\cdot}6\times10^8$		$1{\cdot}3\times10^7$
			$5{\cdot}2\times10^6$		$3{\cdot}2\times10^7$
		Coli-aerogenes	$5{\cdot}3\times10^6$		$2{\cdot}4\times10^6$
			$1{\cdot}5\times10^6$		$5{\cdot}8\times10^6$
			$7{\cdot}4\times10^6$		$4{\cdot}8\times10^6$

* Each count comes from a separate bird. †'Bacteroides' counts minimal owing to faulty technique. nt, not tested; nf, not found.

that these isolates are different from all organisms hitherto reported in currently accepted classifications of Gram-negative anaerobic rods. A detailed study of their identification will be reported elsewhere.

The 'bacteroides' and coli-aerogenes in chickens fed on control and antibiotic supplemented feeds

It was evident from the above tests that 'bacteroides' were present in the caeca of both young and older birds. Table 4 shows both the coli-aerogenes and bacteroides counts obtained in two experiments in which birds were fed with and without chlortetracycline supplements throughout life. Unfortunately, although the feeding experiments were commenced several times, no reliable numbers could be obtained for 17 day old birds. In the final experiment (Table 4, experiment 2), using the technique described in the Methods section, 10^7–10^8 'bacteroides'/g were found in the 12 week old birds. The true figures may be much higher, however, because EVA agar was used for their isolation. In the first experiment the same groups of birds were sampled at 17 days and at 7, 8 and 13 weeks. Although no exact reliance can be placed on the 'bacteroides' counts, the organisms were found to be present on almost every occasion and no difference was detected between the numbers in the control and in the antibiotic fed birds. This result was confirmed in the second experiment where 2 groups of birds had been fed for 12 weeks on control feed and feed containing 100 μg of chlortetracycline/g. In every case coli-aerogenes counts were made at the same time and in respect of these also there was no difference between the control and antibiotic fed birds.

Clortetracycline resistance. It was shown previously by Gordon *et al.* (1959) that coli-aerogenes bacteria isolated from birds reared on diets containing chlortetracycline were more resistant to the antibiotic; hence tests were made of the antibiotic sensitivity of these organisms isolated in these experiments. The MacConkey's agar plates used for counting the coli-aerogenes bacteria were replicated on Bacto heart infusion agar containing 1 or 10 μg of chlortetracycline/ml. In every case a clear difference in resistance was obtained between organisms from the control and the antibiotic fed birds. None of the organisms from the control birds grew on agar containing chlortetracycline at 1 μg/ml, whereas all the organisms from the birds fed on the diets supplemented with chlortetracycline at 25 or 100 μg/g grew on agar containing 10μg of the antibiotic/ml.

With the 'bacteroides' the position was different and the results are shown in Table 5. Although very few replica plates were made during experiment 1 there was no difference between antibiotic fed and control birds. In experiment 2 all the 'bacteroides' from the antibiotic fed birds grew on RCM agar containing 10 μg of chlortetracycline/ml, but some of the 'bacteroides' from the control birds also grew in the presence of this concentration.

The 'bacteroides' isolated from the chickens were purified and after several further subcultures were again tested for resistance to chlortetracycline. The results, given in Table 6, indicate that there was now no real difference between the strains isolated from the control and antibiotic supplemented birds.

Table 5. *The chlortetracyline (CTC) resistance of freshly isolated strains of 'bacteroides' from birds reared on diets with and without CTC*

Expt. no.	Age of birds (weeks)	Level of CTC in diet (µg/g)	Nos. of colonies tested*	Nos. of colonies resistant to CTC†	
				1 µg/ml	10 µg/ml
1	7	0	10	10	‡
			14	14	‡
		100	20	3	‡
	8	0	4	3	0
			4	4	0
		100	50	0	0
	13	25	38	15	0
			24	15	0
2	12	0	20	5	0
			13	13	6
			11	11	0
		100	6	6	6
			5	5	5
			4	4	4

* These colonies were on the highest dilution plate of the original EVA agar count which showed growth. Each group of colonies came from a separate bird.

† Judged by ability to grow when replicated from the original EVA agar count plate on to RCM agar containing CTC at the stated concentration.

‡ Not tested.

Table 6. *The chlortetracycline (CTC) resistance of pure cultures of 'bacteroides' isolated from chickens reared on diets with and without CTC*

Expt. no.	Level of CTC in diet (µg/g)	No. of birds	No. of strains tested	No. of strains resistant* to CTC at (µg/ml)			
				0	0·1	1·0	10
1	0	3	5	5	3§	3	0
	25	3	23	23	23	20	7
	100	4	8	8	8	8	0
2	0	3	34	34	34	26	13
	100	3	31	31	31	26†	19‡

* Able to grow on RCM agar containing the specified CTC concentration.

† When the test was repeated after further subcultures one of these strains grew only at 0·1 µg/ml.

‡ When the test was repeated after further subcultures four of these strains failed to grow at 10 µg/ml.

§ The two sensitive strains were gas producers, possibly *Sphaerophorus* spp.

DISCUSSION

Although many methods and selective media for the isolation of the Gram-negative obligate anaerobes have been published, they are apparently unsuitable for conditions where the 'bacteroides' may be considerably outnumbered by coli-aerogenes bacteria. The organisms isolated in the experiments described above closely resembled the coli-aerogenes organisms in their sensitivity to the various inhibitors tested. Beerens (1957) found that he could incorporate $1:10^4$ of sodium azide in his media for the isolation of either *Ristella*, *Sphaerophorus* or *Fusiformis* spp., but this concentration was too inhibitory for the chicken isolates when used with RCM agar or broth.

The ethyl violet–azide agar eventually used in these experiments probably isolated only a few of the types present in the caeca of chickens, as several standard Gram-negative anaerobic strains tested failed to grow on this medium. Nevertheless 'bacteroides' were found at levels of 10^7–10^8/g and must therefore be considered as one of the major groups present in the caeca of normal chickens. The identification of these organisms is still under investigation. They are closely related to the *Ristella* strains of Beerens but differ in certain physiological characteristics.

It was shown previously that when chlortetracycline was fed to chickens at 25 μg/g of feed continuously for 12 weeks or at 400 μg/g intermittently for fortnightly periods, the faecal streptococci isolated were about 100 times more resistant to that antibiotic than those from the control chickens, and that a different species predominated, the strains being all of a single serological type (Barnes, 1958; Elliott & Barnes, 1959). It was also shown by Gordon *et al.* (1959) that the coli-aerogenes bacteria isolated from the antibiotic fed birds were much more resistant, a result again confirmed by these experiments. The results for 'bacteroides' described in this paper indicate that the response of these organisms to the antibiotic is different. Replica plating directly from the original isolation plates indicated that the colonies from the antibiotic fed birds had a slightly higher resistance to the antibiotic than the control birds. When the organisms isolated from the control and antibiotic fed birds were purified and tested again after several subcultures no real difference could be found between the two groups. It is also significant that when some of the more resistant cultures isolated from the antibiotic fed groups were retested it was found that their resistance was lower than before. It appears that the 'bacteroides' may have obtained a temporary resistance which was lost on further subculture in the absence of the antibiotic. Preliminary tests have also indicated little difference between the types of 'bacteroides' isolated in the two groups.

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REFERENCES

BAIRD-PARKER, A. C. (1955). Isolation of *Leptotrichia buccalis* and *Fusobacterium* species from oral material. *Nature, Lond.*, **180**, 1056.

BARNES, E. M. (1956). Methods for the isolation of faecal streptococci (Lancefield group D) from bacon factories. *J. appl. Bact.* **19**, 193.

BARNES, E. M. (1958). The effect of antibiotic supplements on the faecal streptococci (Lancefield group D) of poultry. *Brit. vet. J.* **114**, 333.

BARNES, E. M. & SHRIMPTON, D. H. (1957). Causes of greening of uneviscerated poultry carcases during storage. *J. appl. Bact.* **20**, 273.

BEATTIE, J. & SHRIMPTON, D. H. (1958). Surgical and chemical techniques for *in vivo* studies of the metabolism of the intestinal microflora of domestic fowls. *Quart. J. exp. Physiol.* **43**, 399.

Beerens, H. (1953–54). Amélioration des techniques d'étude et d'identification des bactéries anaérobies. *Ann. Inst. Pasteur Lille* **6**, 36.

Beerens, H. (1957). Milieux sélectifs pour l'isolement de quelques espèces de bactéries anaérobies à Gram-négatif. *Ann. Inst. Pasteur Lille* **9**, 86.

Beerens, H. & Castel, M. M. (1960). Action de la bile sur la croissance de certaines bactéries anaérobies à Gram-négatif. *Ann. Inst. Pasteur* **99**, 454.

Breed, R. S., Murray, E. G. D. & Smith, N. R. (1957). Bergey's *Manual of Determinative Bacteriology*, 7th ed. London: Ballière, Tindall & Cox.

Eggerth, A. H. & Gagnon, B. H. (1933). The bacteroides of human faeces. *J. Bact.* **25**, 389.

Elliott, S. D. & Barnes, E. M. (1959). Changes in the serological type and antibiotic resistance of Lancefield group D streptococci in chickens receiving dietary chlortetracycline. *J. gen. Microbiol.* **20**, 426.

Finegold, S. M. & Hewitt, W. L. (1955–6). Antibiotic sensitivity pattern of *Bacteroides* species. In *Antibiotics Annual*, 1955–6, p. 794. New York: Medical Encyclopaedia Inc.

Garside, J. S., Gordon, R. F. & Tucker, J. F. (1960). The emergence of resistant strains of *Salmonella typhimurium* in the tissues and alimentary tracts of chickens following the feeding of an antibiotic. *Res. vet. Sci.* **1**, 184.

Gordon, R. F., Garside, J. S. & Tucker, J. F. (1959). Emergence of resistant strains of bacteria following the continuous feeding of antibiotics to poultry. *Int. vet. Congr., Madrid*, **2**, 347.

Hine, M. K. & Berry, G. P. (1937). Morphological and cultural studies of the genus *Fusiformis*. *J. Bact.* **34**, 517.

Lederberg, J. & Lederberg, E. M. (1952). Replica plating and indirect selection of bacterial mutants. *J. Bact.* **63**, 399.

Lewis, K. H., Bedell, M. & Rettger, L. F. (1940). Non-sporulating anaerobic bacteria of the intestinal tract. II. Growth facilitating factors. *J. Bact.* **40**, 39.

Mabbitt, L. A. & Zielinska, M. (1956). The use of a selective medium for the enumeration of lactobacilli in Cheddar cheese. *J. appl. Bact.* **19**, 95.

Mossel, D. A. A. (1959). The suitability of certain obligate anaerobic nonsporeforming enteric bacteria, as part of a more extended bacterial association, as indicators of faecal contamination in foods. *J. appl. Bact.* **22**, 184.

Riemann, H. (1957). Some observations on the germination of *Clostridium* spores and the subsequent delay before the commencement of vegetative growth. *J. appl. Bact.* **20**, 404.

Stevens, W. C. & Harrison, A. P. (Jr.) (1958). *In vitro* antibiotic sensitivities of *Bacteroides* and similar forms. *Antibiotics & Chemotherapy* **8**, 192.

Slanetz, L. W. & Rettger, L. F. (1933). A systematic study of the fusiform bacteria. *J. Bact.* **26**, 599.

Weiss, J. E. & Rettger, L. F. (1937). The Gram-negative bacteroides of the intestine. *J. Bact.* **33**, 423.

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Thomas, S. B., Hobson, P. M., Bird, E. R., King, K. P., Druce, R. G. & Cox, D. R. (1962). *J. appl. Bact.* **25** (1), 107–115.

THE MICROFLORA OF RAW MILK AS DETERMINED BY PLATING ON YEASTREL-MILK AGAR INCUBATED AT 30°

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SUMMARY: The classification of the predominant types of bacteria found in 87 farm milk supplies, examined within 8–10 hr of production, was done by plating on Yeastrel-milk agar, incubating for 72 hr at 30° and picking 24 colonies at random from each countable plate for detailed study. Although the results indicate some general trends, such as the predominance of micrococci in milk of low count ($<2\times10^4$ colonies/ml), the co-dominance of streptococci, corynebacteria and micrococci in milk with colony counts between 2×10^4 and 2×10^5/ml, and the dominance of nonpigmented Gram-negative rods and streptococci in the high count milks (2×10^5–5×10^6 colonies/ml), several milk samples, irrespective of colony count level, had a characteristic individual microflora dominated by either corynebacteria, aerobic sporeformers, coli-aerogenes organisms, fluorescent pseudomonads or flavobacteria.

The main purpose of this investigation, carried out at Aberystwyth, Bangor and Cardiff during a period of 14 months, was to ascertain the distribution of the prevalent types of milk bacteria forming colonies on Yeastrel-milk agar (YMA) incubated at 30° for 72 hr. The milk was plated between 8 and 10 hr of production, as is normally done in advisory work. Previous work of this kind has been of a specialist or restricted nature. Thus, Wilson *et al.* (1935) studied the bacterial flora at the time of methylene blue reduction in milks incubated at various temperatures, Garvie & Rowlands (1952) investigated the distribution of the various types of bacteria in 12 raw milk samples held overnight at 14°, and Carreira, Clegg, Clough & Thiel (1955) confined their investigation to milk produced under extremely good experimental conditions. The most comprehensive investigation of the development of bacterial populations in milk to date is that of Gibson & Abd-el-Malek (1957). This included aseptically drawn milk samples, single farm milk samples in which the udder organisms were still prominent, and farm milks containing a denser and more complex microflora.

The present investigation was designed to provide data which could be considered representative of the dominant organisms in the microflora of farm milk supplies at the time of examination for advisory purposes.

Materials and Methods

Samples (3 oz) of morning's milk were taken directly from milk cans at 87 farms, plated within 8–10 hr of milking on YMA and incubated at 30° for 72 hr. Approximately 24 colonies were picked at random, using a Harrison disc, from each countable plate (30–300 colonies) into yeast-dextrose-Lemco broth (YDB) and incubated at 30° for 24–72 hr. Each YDB culture was either streaked on the surface of a dried YMA plate, or high dilutions of it were plated on poured YMA plates. After incubation of the YMA plates at 30° for 24–48 hr a well isolated colony was picked on to two YMA slopes, one of which was incubated at 30° and the other at 22°. No further purification was carried out, and the few mixed cultures which resulted were included in the unclassified group.

The YMA slopes provided culture material for the following differential tests: Gram-reaction, by Dowson's modification (Report, 1949), and morphology were determined by examination of 30° cultures in 24–30 hr, and when large Gram-positive rods were observed, a methyl violet stained preparation of the same culture, after further incubation for 48–72 hr, was examined for the presence of spores; the catalase test, according to a microscopical slide modification of the method described by Wilson & Miles (1955), was applied to 72–96 hr old cultures incubated at 30°; cultures composed of nonpigmented, Gram-negative rods were inoculated into MacConkey's broth incubated at 30°, and the formation of acid and gas was recorded in 5 days; the YMA slope cultures incubated at 22° were inoculated into litmus milk, incubated at 22°, and the type of fermentation occurring in 14 days was recorded; the 22° YMA slope cultures were examined for development of pigment after 14 days and the kind of pigmentation recorded.

The criteria obtained from the above tests were used for distinguishing the main types of bacteria according to Fig. 1.

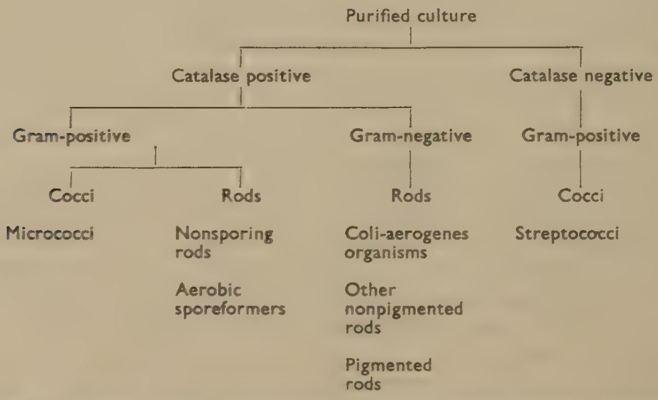
Purified culture
Catalase positive
Catalase negative
Gram-positive
Gram-negative
Gram-positive
Cocci
Rods
Rods
Cocci
Micrococci
Nonsporing rods
Aerobic sporeformers
Coli-aerogenes organisms
Other nonpigmented rods
Pigmented rods
Streptococci

Fig. 1. Scheme for the differentiation of the main types of bacteria isolated from milk.

Cultures of streptomycetes do not fit into the above scheme, but were distinguished by their characteristic 'chalk-powder' growth on YMA slopes, together with the branched mycelium with chains of terminal conidia in stained microscopical preparations 'dug out' of the agar.

Many of the cultures of asporogenous Gram-positive rods were corynebacteria (microbacteria), which could be distinguished by their 'chinese-writing' appearance under the microscope and by the small colonies which generally developed a yellow pigment.

Cultures of Gram-negative rods which formed acid and gas in MacConkey's broth within 5 days at 30° were considered to be coli-aerogenes organisms, the anaerogenic strains which developed a yellow, green or blue fluorescent water soluble pigment were classified as fluorescent pseudomonads, and those which formed yellow or orange water insoluble pigment, as flavobacteria. The unpigmented, anaerogenic Gram-negative rods would include *Achromobacter* spp., *Alcaligenes* spp., nonfluorescent pseudomonads and anaerogenic paracolon strains.

RESULTS AND DISCUSSION

The distribution of milk samples in relation to the range of colony count (Table 1) is representative of the kind of results obtained during advisory work. Farm milk supplies giving colony counts of $<2\times10^4$/ml at 30° are considered to have been produced and handled under satisfactory conditions (Griffiths, Druce & Thomas, 1957), and counts $<5\times10^3$ are not uncommon where the milking equipment is efficiently cleansed. When the colony counts range from 2×10^4 to 2×10^5 it is generally recognized that cleansing methods could be improved, whereas counts of $>2\times10^5$ are termed unsatisfactory. Milk giving a colony count of more than 10^6/ml has obviously been produced under very unsatisfactory conditions or handled in unsterile equipment.

The distribution of the 2,065 bacterial cultures indicates that representative numbers have been derived from each of the five ranges of colony count, so that the microflora studied depicts the occurrence of the predominant types found in raw milk produced under commercial conditions when examined by means of colony count at 30° within 8–10 hr of milking.

Table 1. *The incidence of milk samples* (a) *and bacterial cultures isolated therefrom* (b) *in relation to range of colony count*

Ranges of colony count/ml*	Numbers and percentages† (a)	(b)
$<5\times10^3$	11 (12·6)	259 (12·5)
5×10^3–2×10^4	24 (27·6)	567 (27·5)
$>2\times10^4$–2×10^5	31 (35·6)	737 (35·7)
$>2\times10^5$–10^6	9 (10·3)	213 (10·3)
$>10^6$–5×10^6	12 (13·9)	289 (14·0)
Totals	87	2,065

* Counts done on YMA incubated at 30° for 72 hr.

† Percentages in brackets.

The microflora of the 11 samples of milk produced under very good hygienic conditions (colony counts of $<5\times10^3$/ml) was dominated by micrococci, which constituted close on 70% of the 259 cultures (Table 2). In the fresh milk examined by Carreira *et al.* (1955) micrococci formed 73–97% of the microflora when hot detergent chlorine wash or immersion cleaning in caustic soda was used for cleansing milking equipment. Staphylococci were also dominant in the farm bottled milk examined by Gibson & Abd-el-Malek (1957).

The only other organisms present in any appreciable numbers were streptococci and asporogenous Gram-positive rods, the latter mainly coryneform types.

Table 2. *The incidence of different types of bacteria according to range of colony count of milk sample*

Types of organisms	Nos. and percentages* of cultures isolated from samples with colony counts† in the ranges					Total cultures
	$<5 \times 10^3$	5×10^3–2×10^4	$>2 \times 10^4$–2×10^5	$>2 \times 10^5$–10^6	$>10^6$–5×10^6	
Micrococci	178 (68·7)	297 (52·4)	219 (29·7)	44 (20·7)	55 (19·0)	793 (38·4)
Streptococci	34 (13·1)	80 (14·1)	125 (17·0)	14 (6·6)	61 (21·1)	314 (15·2)
Asporogenous Gram +ve rods	26 (10·0)	55 (9·7)	108 (14·6)	27 (12·7)	10 (3·5)	226 (11·0)
Coli-aerogenes bacteria	0 (0·0)	5 (0·9)	66 (9·0)	56 (26·3)	8 (2·8)	135 (6·5)
Pigmented Gram −ve rods	4 (1·6)	21 (3·7)	99 (13·4)	51 (23·9)	26 (9·0)	201 (9·7)
Nonpigmented Gram −ve rods	11 (4·3)	19 (3·3)	66 (9·0)	14 (6·6)	122 (42·2)	232 (11·2)
Aerobic spore-forming rods	5 (1·9)	70 (12·3)	31 (4·2)	2 (0·9)	3 (1·0)	111 (5·4)
Streptomycetes	0 (0·0)	3 (0·5)	3 (0·4)	0 (0·0)	0 (0·0)	6 (0·3)
Unclassified	1 (0·4)	17 (3·1)	20 (2·7)	5 (2·3)	4 (1·4)	47 (2·3)
Total cultures	259	567	737	213	289	2,065

* Percentages in brackets. † Counts done on YMA incubated at 30° for 72 hr.

The microflora of the next group of 24 samples with reasonably low colony counts (5×10^3 to 2×10^4/ml) was not very different from that of the first group. Micrococci were not so dominant, forming only 52% of the cultures, and aerobic sporing rods were much more common (12%). Cultures of coli-aerogenes organisms were isolated from only two samples; other types of Gram-negative rods were found in small numbers in 14 samples.

The series of 31 samples of doubtful hygienic quality, with colony counts between 2×10^4 and 2×10^5/ml, had a much more complex microflora which did not show any standard pattern. Micrococci now constituted barely a third of the 737 strains, whereas streptococci, asporogenous Gram-positive rods, coli-aerogenes organisms and other types of Gram-negative rods showed an appreciable increase. Examination of the distribution of different types of bacteria in individual samples showed that single types were dominant in 24 samples. Thus micrococci were dominant in six samples, streptococci in four, asporogenous Gram-positive rods in three, coli-aerogenes organisms in four, pigmented Gram-negative rods in three, anaerogenic, nonpigmented Gram-negative rods in three and aerobic sporeformers in one. In the remaining seven samples, micrococci, streptococci and anaerogenic Gram-negative rods constituted about a third each.

Micrococci (21%), coli-aerogenes organisms (26%) and pigmented Gram-negative rods (24%), together with coryneform types (13%) were commonly found among the 289 strains isolated from the nine milk samples with high colony counts ($>2\times10^5$–10^6/ml). Micrococci were dominant in one sample, coli-aerogenes organisms in two and fluorescent pseudomonads in two, whereas micrococci, coli-aerogenes organisms, coryneforms and pigmented Gram-negative rods (flavobacteria and fluorescent pseudomonads) were about equally represented in each of the four remaining samples. Streptococci were isolated from five samples, but occurred in appreciable numbers in only one sample.

Nonpigmented Gram-negative rods, members of the genera *Achromobacter*, *Alcaligenes* or *Pseudomonas* (nonfluorescent), were particularly dominant in the 12 milks with very high colony counts ($>10^6$–5×10^6/ml), constituting 42% of the 289 cultures. Streptococci showed an appreciable increase, now forming 21% of the cultures, whereas micrococci were still present in quite significant numbers, but were dominant, as were streptococci and pigmented Gram-negative rods (*Flavobacterium* spp.), in only one sample. On the other hand, anaerogenic nonpigmented Gram-negative rods were dominant in five samples, constituting 75% of the strains isolated from two of them. In each of the remaining four samples, nonpigmented Gram-negative rods formed half the strains, with micrococci and streptococci making up the other half. Sporeformers were detected in very few of the high count samples, which agrees with the findings of Carreira *et al.* (1955).

The distribution of the eight main types of bacteria among all the 2,065 cultures provides an interesting comparison with the results obtained by Garvie & Rowlands (1952) for cultures picked from 22° YMA plates poured with 14 hr old milk. These workers found a much lower incidence of micrococci (14% compared with 38%) and a somewhat higher incidence of streptococci (24% compared with 15%); Gram-positive rods were also more common (18% compared with 11%) but, as in the present investigation, they were nearly all corynebacteria. Nonpigmented Gram-negative rods, which they listed as achromobacters, were very dominant, constituting 31% of the microflora compared with 11% in the present investigation. Such a general summary, however, does not show the complex pattern of distribution which is so evident when the microfloras of the five groups of milk samples are considered separately and still more evident in the microfloras of individual milk samples.

Milk samples containing different types of bacteria. The number and percentage of milk samples in which the different types of bacteria were detected, and the proportion in which the different types constituted 50% or more of the microflora, i.e. in which they were frequent or dominant, are given in Table 3. Micrococci, streptococci and asporogenous Gram-positive rods (mainly coryneform types) were found in a high proportion of milks.

Micrococci were detected in 91% of samples and they constituted an appreciable proportion of the microflora in 44%. This type is evidently present in most raw milk supplies; Innes (1956), Tee (1957) and Walters (1959) recovered coagulase positive staphylococci from a high proportion of farm milk supplies, and it is significant that McKenzie & Hunter (1961) found micrococci to be the main survivors on cows' teats after washing the udders prior to milking. Gibson & Abd-el-Malek (1957) reported that staphylococci formed a dominant group in most of the uncontaminated milks, and that they were present in most of the samples of bulk raw milk taken from creamery storage tanks.

Table 3. *The incidence of milk samples in which various bacterial types were detected* (a), *and in which these types constituted* 50% *or more of the microflora* (b)

Bacterial types	Nos. and percentages* of samples (a)	(b)
Micrococci	79 (90·8)	38 (43·7)
Streptococci	67 (77·0),	10 (11·5)
Asporogenous Gram +ve rods	56 (64·3)	4 (4·6)
Coli-aerogenes bacteria	17 (19·5)	6 (6·9)
Pigmented Gram −ve rods	45 (51·7)	6 (6·9)
Nonpigmented Gram −ve rods	46 (52·9)	10 (11·5)
Aerobic spore-forming rods	26 (29·8)	4 (4·6)
Streptomycetes	5 (5·7)	0 (0·0)
Unclassified	28 (32·1)	0 (0·0)
Total milk samples examined	87	87

* Percentages in brackets.

Though streptococci were detected in 77% of the samples they were frequent or dominant in only 11%. Gibson & Abd-el-Malek (1957) found *Streptococcus lactis* and *Strep. faecalis* in practically all the bulk milk samples, but they were rarely detected in milk bottled on the farm. Gram-positive, nonsporing rods (mainly coryneform types in morphology) were found in 64% of the samples, but they were frequent in only 5%. No distinction was made between the heat labile diphtheroid types and the thermoduric corynebacteria (microbacteria), as no heat resistance tests were done.

Coli-aerogenes organisms were found in 19% of the milk samples. They constituted an appreciable proportion of the colonies picked from 7% of the samples. On the other hand, the presumptive coli-aerogenes test at 30° showed their presence in 10^{-3} ml or less in 25% of the milk samples. This illustrates what is obvious in this type of survey: most of the types of bacteria selected by the colony picking technique must constitute an appreciable proportion of the microflora of the milk able to form colonies on YMA at 30°, i.e. only the more abundant types are selected.

Pigmented Gram-negative rods (mainly fluorescent pseudomonads and flavobacteria) were found in about half the milk samples, and they were frequent to dominant in 7%. These would be predominantly psychrophilic types able to multiply at relatively low temperatures, and it is of interest to observe that Wilson *et al.* (1935) found that they were some of the most frequently occurring organisms in raw milk at the time of methylene blue reduction at 17·5°.

Nonpigmented Gram-negative rods were found in 52% of the samples, and they formed an appreciable proportion of the microflora in 11%. Wilson *et al.* (1935) found that the organisms most common in raw milk at the time of reduction of methylene blue at 17·5° belonged to the *Achromobacter* group, whereas Gibson & Abd-el-Malek (1957) found *Alcaligenes* spp. in most of the samples of bulk milk and high count farm milk supplies. *Achromobacter* spp. constituted a very small proportion (1–12%) of the initial microflora of the carefully produced milk examined by Carreira *et al.* (1955), but these organisms showed a considerable increase (11% to 31%) in the microflora of the same milk after it had been aged for 24 hr at 22°.

Aerobic sporeformers were isolated from 30% of the samples, but they constituted an appreciable proportion of the microflora in only 5%. This confirms the results reported by Ridgway (1955), who found that 12% of the farm milk supplies examined during winter, when the cows were housed and sporeformers were most frequent, gave high spore counts (>100/ml).

Table 4. *The incidence of different types of organisms giving various reactions in litmus milk after* 14 *days at* 22°

Types of organisms	No. of cultures	Nos. and percentages (in brackets) of cultures giving					
		Acid clot	Acid only	Alkali	Proteolysis	Reduction only	No change
Micrococci	793	200 (25·2)	183 (23·1)	21 (2·7)	112 (14·1)	72 (9·1)	205 (25·8)
Streptococci	314	202 (64·3)	90 (28·7)	0 (0·0)	9 (2·9)	4 (1·2)	9 (2·9)
Asporogenous Gram +ve rods	226	13 (5·8)	64 (28·3)	32 (14·1)	53 (23·5)	17 (7·5)	47 (20·8)
Coli-aerogenes bacteria	135	84 (62·2)	25 (18·5)	0 (0·0)	6 (4·5)	20 (14·8)	0 (0·0)
Pigmented Gram −ve rods	201	16 (8·0)	15 (7·5)	3 (1·4)	117 (58·2)	11 (5·5)	39 (19·4)
Nonpigmented Gram −ve rods	232	18 (7·8)	14 (6·0)	41 (17·6)	110 (47·4)	18 (7·8)	31 (13·4)
Aerobic spore-forming rods	111	8 (7·2)	1 (0·9)	8 (7·2)	68 (61·3)	9 (8·1)	17 (15·3)
Streptomycetes	6	1 (16·6)	0 (0·0)	1 (16·6)	2 (33·4)	0 (0·0)	2 (33·4)
Unclassified	47	11 (23·4)	2 (4·3)	2 (4·3)	18 (38·3)	9 (19·1)	5 (10·6)
Total cultures	2,065	553 (26·8)	394 (19·1)	108 (5·3)	495 (23·9)	160 (7·7)	355 (17·2)

Fermentation of litmus milk. The reactions produced in litmus milk after incubation for 14 days at 22° are given in Table 4. There were two main groups of micrococci, nearly half the cultures forming acid and most of the remainder being inert or proteolytic. This shows some similarity with the two subgroups of dairy micrococci (group III) outlined by Abd-el-Malek & Gibson (1948). A high proportion of the streptococci and coli-aerogenes organisms formed an acid clot. Some of the latter peptonized and digested the milk, thus resembling the proteolytic strains of anaerogenic *Citrobacter* and *Klebsiella cloacae* reported by Thomas (1955).

Although the majority of the Gram-positive, nonsporing rods resembled corynebacteria in morphology, their reactions in litmus milk at 22° differed somewhat from those reported by Thomas & Thomas (1955) for thermoduric corynebacteria isolated from milk cans. A high proportion of the latter formed an acid clot in 14 days, whereas more than a third of the former were proteolytic or formed alkali, a third were relatively inert and most of the remainder were weak acid producers.

Close on 60% of the pigmented Gram-negative rods were strongly proteolytic, but some (15%) gave acid reactions. These results agree with those reported by Thomas (1949) and Jones & Thomas (1950) for psychrophilic strains of *Pseudomonas fluorescens* and *Flavobacterium arborescens* from farm water supplies. More recently, Druce & Thomas (1961, unpublished data) found that two-thirds of the 44 strains of *Flavobacterium* isolated from milk and water were strongly proteolytic, but a quarter of the strains formed acid and curdled the milk within 14 days at 22°.

The nonpigmented Gram-negative rods showed considerable variation in their action in litmus milk. Some showed no change; more often the milk turned alkaline or was peptonized and sometimes it became acid and curdled. Although they may have included strains of *Achromobacter*, nonfluorescent *Pseudomonas* and *Alcaligenes* spp., it is interesting to note that these are the litmus milk reactions of *Achromobacter* spp. according to Wilson & Miles (1955).

The majority of the aerobic sporeforming rods were strongly proteolytic, 23% were relatively inert, and 7% formed an acid clot resembling the lactose fermenting variants of *Bacillus cereus* described by Robertson, Yale & Breed (1926), Smith Gordon & Clark (1946) and Egdell & Bird (1950).

REFERENCES

Abd-el-Malek, Y. & Gibson, T. (1948). Studies in the bacteriology of milk. II. The staphylococci and micrococci of milk. *J. Dairy Res.* **15**, 249.

Carreira, D. F. C., Clegg, L. F. L., Clough, P. A. & Thiel, C. C. (1955). The effect of steam, hypochlorite and caustic soda used for treating direct-to-can milking equipment, on the bacteriological quality and flora of milk. *J. Dairy Res.* **22**, 166.

Egdell, J. W. & Bird, E. R. (1950). The types of organisms present in commercially pasteurized milk. *J. Soc. Dairy Tech.* **3**, 171.

Garvie, E. I. & Rowlands, A. (1952). The role of micro-organisms in dye reduction and keeping quality tests. I. The change in dominant bacterial flora during incubation at 37·5° and 22° and the part played by staphylococci and the coli-aerogenes group. *J. Dairy Res.* **19**, 133.

Gibson, T. & Abd-el-Malek, Y. (1957). The development of bacterial populations in milk. *Canad. J. Microbiol.* **3**, 203.

Griffiths, D. G., Druce, R. G. & Thomas, S. B. (1957). Advisory microbiological standards and tolerances for raw milk. *J. appl. Bact.* **20**, 243.

Innes, A. G. (1956). Coagulase positive staphylococci from bulk milk supplies low in solids-not-fat. *J. appl. Bact.* **19**, 39.

Jones, G. E. & Thomas, S. B. (1950). The occurrence of a *Flavobacterium* species in chilled water. *Proc. Soc. appl. Bact.* **13**, 13.

McKenzie, D. A. & Hunter, A. C. (1961). Further studies on the disinfection of cows' teats prior to milking. *J. appl. Bact.* **24**, 71.

Report (1949). The classification of the coli-aerogenes bacteria. Report of the Coliform Sub-Committee. *Proc. Soc. appl. Bact.* (2), 3.

Ridgway, J. D. (1955). Some recent observations on the bacteriology of sterilized milk. *J. appl. Bact.* **18**, 374.

ROBERTSON, A. H., YALE, M. W. & BREED, R. S. (1926). Non-thermophilic, spore-forming bacteria associated with pasteurizing equipment. *Tech. Bull. N.Y. St. agric. Exp. Sta.* no. 119.

SMITH, N. R., GORDON, R. E. & CLARK, F. E. (1946). Aerobic mesophilic spore-forming bacteria. *Misc. Publ. U.S. Dep. Agric.* no. 559.

TEE, G. H. (1957). The incidence of penicillin-resistant strains of *Staphylococcus aureus* in dairy herd samples. *Mon. Bull. Ministr. Hlth Lab. Serv.* **16**, 141.

THOMAS, S. B. (1949). The types of bacteria commonly found in farm and creamery water supplies and their action on milk and milk products. *J. Soc. Dairy Tech.* **2**, 224.

THOMAS, S. B. (1955). Coli-aerogenes bacteria in raw milk. *J. appl. Bact.* **18**, 331.

THOMAS, S. B. & THOMAS, B. F. (1955). Thermoduric microbacteria (corynebacteria) in milk. *Dairy Ind.* **20**, 839.

WALTERS, A. H. (1959). The isolation of coagulase positive staphylococci from routine composite milk samples. *J. appl. Bact.* **22**, 248.

WILSON, G. S. & MILES, A. A. (1955). Topley & Wilson's *Principles of Bacteriology and Immunity*, 4th ed. London: Arnold.

WILSON, G. S., TWIGG, R. S., WRIGHT, R. C., HENDRY, C. B., COWELL, M. P. & MAIER, I. (1935). *The Bacteriological Grading of Milk. Spec. Rep. Ser. med. Res. Coun.* no. 206. London: H.M.S.O.

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FLAGELLATION OF *AZOTOBACTER* SPP. AS DEMONSTRATED BY ELECTRON MICROSCOPY

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SUMMARY: The results of an electron microscope survey of flagellation in the genus *Azotobacter* are presented and the implications of the findings are discussed.

Although individual strains of *Azotobacter* have received considerable attention on account of their diverse biochemical activities and large cell size the genus as a whole is poorly known to the majority of bacteriologists. Its classification, however, presents problems which are of interest in the broader context of bacterial taxonomy and the genus is worthy of more than passing attention. In recent years new azotobacters have been described which, in contrast to the established species, are claimed to be polar flagellate. Work on the genus has been hampered by the absence of a systematic study of flagellation in the motile species. During the past few years we have examined over fifty strains of *Azotobacter* including representatives of each of the known species, using flagella staining techniques, and these have indicated that different types of flagellation do in fact exist. Such observations should be checked by electron microscopy (Hayward & Hodgkiss, 1961) and this paper records the results of an electron microscope survey using shadowing and phosphotungstic acid negative staining techniques.

Methods

Cultures. Twenty *Azotobacter* strains comprising type cultures and recently isolated strains were examined. Cultures were grown on nitrogen-free agar of the following composition (% w/v): glucose, 1; K_2HPO_4, 0·1; $MgSO_4.7H_2O$, 0·02; $CaCO_3$, 0·1; NaCl, 0·02; $Na_2MoO_4.2H_2O$, 0·0005; and agar, 2·0. The components were dissolved in distilled water and the medium was sterilized by autoclaving at 15 lb/in² for 10 min. Slope cultures were incubated at 30° for 36 hr.

Preparation of specimens for electron microscopy. Cultures were harvested in distilled water, stored in suspension overnight in the refrigerator at +5°, washed three or more times in distilled water by slow centrifugation to remove capsular material, and finally resuspended in distilled water.

Shadow cast specimens. Droplets of the suspension were applied to formvar grids, allowed to dry in air and shadow cast with gold-palladium.

Negative stained preparations. A portion of the bacterial suspension was mixed with an equal volume of a 2% (w/v) solution of phosphotungstic acid adjusted to pH 7·4 with N KOH (Brenner & Horne, 1959). Small drops were then placed on formvar films which had been stabilized with carbon, partially blotted to remove excess phosphotungstic acid, and allowed to dry in air.

All preparations were examined in the Siemens Elmiskop I electron microscope using the single condenser system, with a 200μ condenser aperture, a 50μ objective aperture and an accelerating voltage of 60 kV. Suitable fields were photographed at a magnification of 8,000 or 10,000 diameters on Ilford N50 thin film half tone plates.

RESULTS

Provided that steps are taken to remove capsular material, the flagella of *Azotobacter* spp. can be stained by conventional methods quite easily and they were readily visible under the electron microscope. The results obtained were fully in accord with visible light observations on stained material and flagellation type was constant for the various strains of each species. The illustrations show typical results.

Peritrichous flagellation was seen with *Azotobacter vinelandii* (Plate 1, a and b), *A. agilis* (Plate 1, c and d) and *A. chroococcum* (Plate 1, e and f). *A. chroococcum* had, as a rule, fewer flagella than the other species. *A. insigne* showed a polar arrangement of up to twelve flagella, which often appeared to arise from two distinct sites at one end of the cell (Plate 2, a and b). *A. macrocytogenes* occurs in two phases (Jensen, H. L., 1955) and both showed polar flagellation with one, or sometimes two, flagella (Plate 2, c and d). *A. beijerinckii* is nonmotile and the strains which we have studied have no flagella.

DISCUSSION

There is a great deal of confusion in the early literature on the genus *Azotobacter* as regards the type of flagellation encountered and the need to clarify the position for taxonomic purposes led Hofer (1944) to study cultures of *A. chroococcum*, *A. beijerinckii*, *A. agilis* and *A. vinelandii*—the four species then known—by direct staining and electron microscopy. He concluded that all of these organisms were peritrichous and peritrichous flagellation has, as a result, become established as one of the characteristics of the genus (cf. Breed, Murray & Smith, 1957).

Derx (1951) described *A. insigne*, which closely resembled *A. agilis* but was apparently polar flagellate. This organism has since been isolated from Danish water courses by Jensen, V. (1955) and by Nagy (pers. comm.) and by one of us (J.R.N.) from a Scottish river. Its flagellation is clearly polar in the sense that flagella are clustered at one end of the cell, and this is reflected in the distinctive, erratic, zigzag and circling type of movement which serves to distinguish this organism from the closely similar but peritrichous *A. agilis*. The consistent appearance of two tufts of flagella with different points of insertion gives an appearance which is not, in our experience, paralleled elsewhere in bacteriology.

A. macrocytogenes was described by Jensen, H. L. (1955), who drew attention to the fact that motile cells were apparently polar flagellate. Cells in the motile or 'M' phase were readily seen to possess one polar flagellum, but motile cells in the 'O' phase were not easy to examine for flagella on account of the large amount of slime associated with the cells. *A. macrocytogenes* is a rare organism. Although subsequently re-isolated from the Danish soil in which it was first found, it has only once been reported from another situation—a soil sample from the Flannan Islands (Norris & Baird, 1960). Both isolates showed one or sometimes two polar flagella in the 'M' and 'O' phases.

The demonstration of organisms with polar flagella in a 'peritrichous' genus, although in conformity with the Adansonian approach to classification, is disconcerting to the traditional taxonomist who believes type of flagellation to be a character of prime importance in bacterial classification. Like Rubenchik (1959) and Leifson (1960) he may be inclined to remove the polar species to other, distant genera. The weight of evidence, however, favours the retention of these six organisms in one closely knit group. All share the characteristics of active nitrogen fixing ability and large cell size. Norris (1960) demonstrated a common antigen present in each of them and a considerable sharing of antigens between some species such as *A. agilis* and *A. insignis*. A study of the saline soluble antigens (Norris & Nagy, 1960) also suggested that the group was a homogeneous one and provided antigenic support for the division into six species which is at present recognized. The close similarity of the polar *A. insignis* and the peritrichous *A. agilis* is emphasized by the finding that several strains labelled *A. agilis* which have existed in culture collections for many years are in fact *A. insignis* (Jensen, V. 1955; Norris & Jensen, 1958).

It is the purpose of this paper to illustrate the flagellation of the genus *Azotobacter* rather than to discuss in detail the classification of the genus, but we would like to record our own view on the taxonomy of these organisms. There are good grounds for dividing the genus *Azotobacter* into two sections, according to ability to produce microcysts (Winogradsky, 1938; Tchan, 1953; Jensen, V. 1955). We would support this and suggest that two genera should be recognized: *Azotobacter*, to include the microcyst-forming species (*Azotobacter chroococcum*, *Azotobacter beijerinckii* and *Azotobacter vinelandii*), and *Azomonas*, to contain the species which do not form microcysts (Winogradsky, 1938; Jensen, V., 1955), in which both peritrichous (*Azomonas agilis*) and polar (*Azomonas insignis* and *Azomonas macrocytogenes*) species would be included.

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REFERENCES

Breed, R. S., Murray, E. G. D. & Smith, N. R. (1957). Bergey's *Manual of Determinative Bacteriology*, 7th ed. London: Baillière, Tindall & Cox.

Brenner, S. & Horne, R. W. (1959). A negative staining method for high resolution microscopy of viruses. *Biochim. biophys. Acta* 34, 103.

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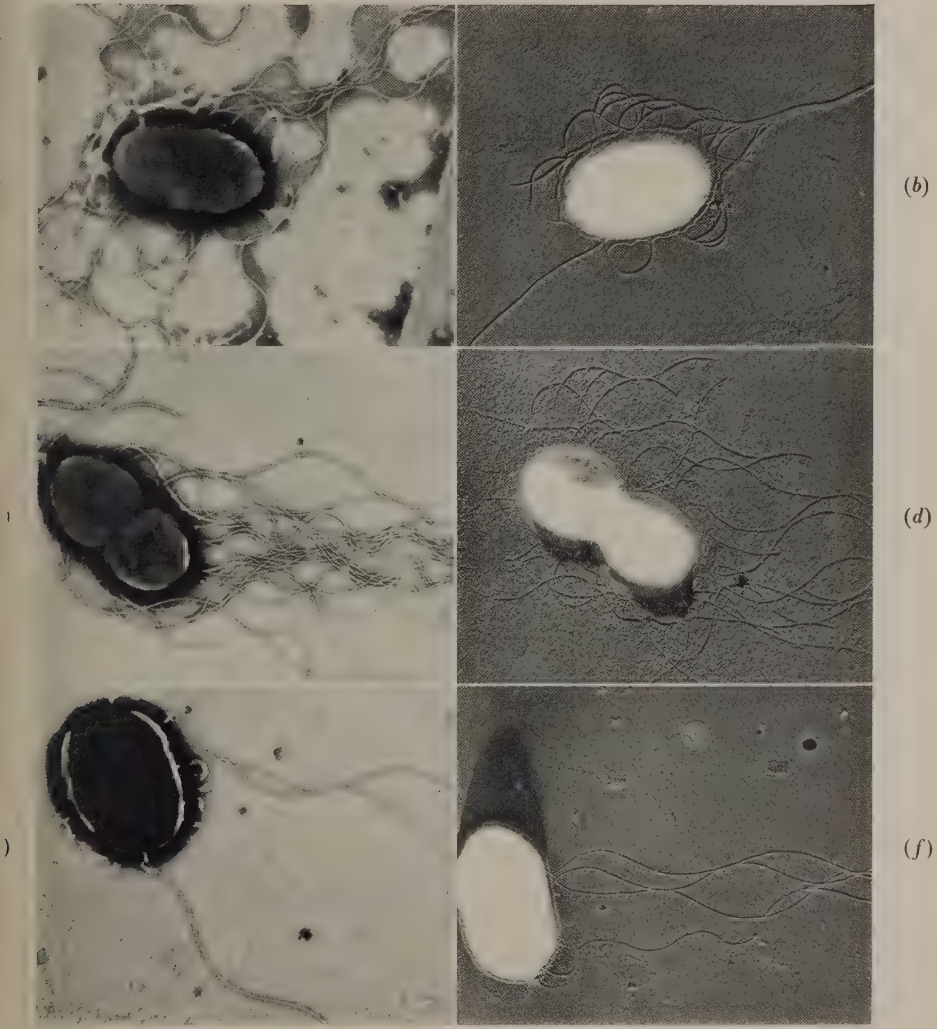

Anne Baillie, W. Hodgkiss and J. R. Norris—Flagellation of *Azotobacter* Spp. as Demonstrated by Electron Microscopy. Plate 1

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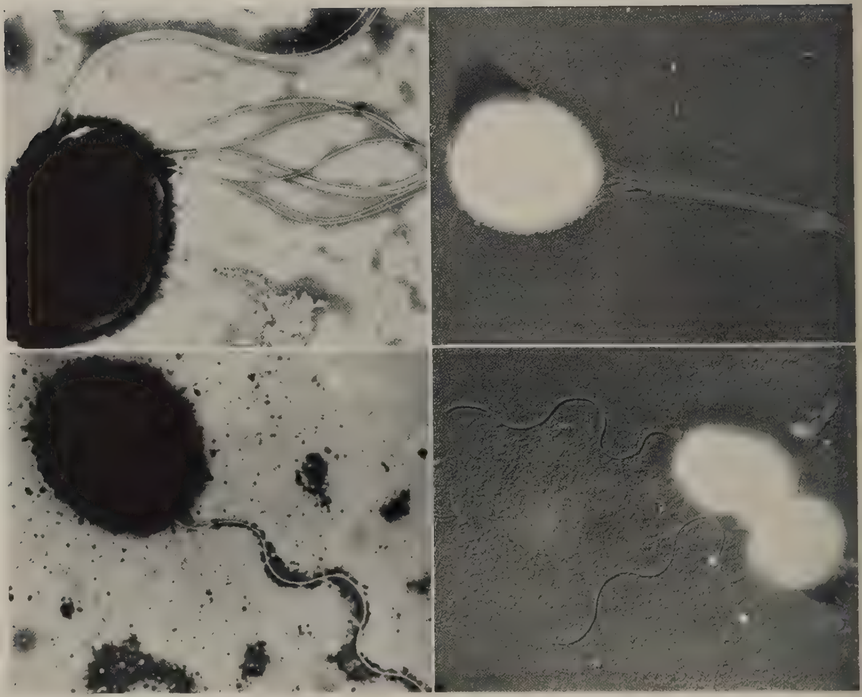

Anne Baillie, W. Hodgkiss and J. R. Norris—Flagellation of *Azotobacter* Spp. as Demonstrated by Electron Microscopy. Plate 2

DERX, H. G. (1951). *Azotobacter insigne* spec. nov., fixateur d'azote à flagellation polaire. *Proc. Acad. Sci. Amst.* **54**, 342.

HAYWARD, A. C. & HODGKISS, W. (1961). Taxonomic relationships of *Xanthomonas uredovorus*. *J. gen. Microbiol.* **26**, 133.

HOFER, A. W. (1944). Flagellation of *Azotobacter*. *J. Bact.* **48**, 697.

JENSEN, H. L. (1955). *Azotobacter macrocytogenes* n. sp., a nitrogen-fixing bacterium resistant to acid reaction. *Acta agric. scand.* **2**, 280.

JENSEN, V. (1955). The *Azotobacter*-flora of some Danish watercourses. *Bot. Tidsskr.* (Copenhagen) **52**, 143.

LEIFSON, E. (1960). *Atlas of Bacterial Flagellation.* London: Academic Press.

NORRIS, J. R. (1960). Antigens of *Azotobacter*. *Nature, Lond.*, **185**, 634.

NORRIS, J. R. & JENSEN, H. L. (1958). Calcium requirements of *Azotobacter*. *Arch. Mikrobiol.* **31**, 198.

NORRIS, J. R. & BAIRD, D. E. (1960). The *Azotobacter* spp. of Flannan Island. *J. appl. Bact.* **23**, x.

NORRIS, J. R. & NAGY, L. (1960). The soluble antigens of *Azotobacter*. *J. appl. Bact.* **23**, ix.

RUBENCHIK, L. I. (1959). A contribution to the systematics of bacteria of the *Azotobacteriaceae* family. *Microbiology, Moscow*, **28**, 309 (English Edition).

TCHAN, Y. T. (1953). Studies of N-fixing bacteria. IV. Taxonomy of genus *Azotobacter* (Beijerinck, 1901). *Proc. Linn. Soc. N.S.W.* **78**, 85.

WINOGRADSKY, S. (1938). Études sur la microbiologie du sol et des eaux. Sur la morphologie et l'oecologie des *Azotobacter*. *Ann. Inst. Pasteur* **60**, 351.

EXPLANATION OF PLATES

PLATE 1. Electron micrographs of *Azotobacter* cells showing flagellation: (a), (c) and (e), phosphotungstic acid preparations; (b), (d) and (f), Au–Pd shadowed preparation, × 12,500 approx.
(a) and (b) *Azotobacter vinelandii.*
(c) and (d) *Azotobacter agilis.*
(e) and (f) *Azotobacter chroococcum.*

PLATE 2. Electron micrographs of *Azotobacter* cells showing flagellation: (a) and (c), phosphotungstic acid preparations; (b) and (d), shadowed preparations, × 12,500 approx.
(a) and (b) *Azotobacter insigne.*
(c) and (d) *Azotobacter macrocytogenes.*

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THE 'VACUUM PACK' METHOD OF PACKAGING FOODS IN RELATION TO THE FORMATION OF THE BOTULINUM AND STAPHYLOCOCCAL TOXINS

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SUMMARY: The development of botulinum toxin in smoked fish and of staphylococcal enterotoxin in packed bacon has been demonstrated. It is to be expected that these toxins may be produced in contaminated commercial products stored under conditions which permit substantial growth of the organisms, though formation of enterotoxin is not a predictable sequel to multiplication of staphylococci.

VACUUM PACKAGING is being used with increasing frequency for specific processed foods. Such foods are marketed in impermeable plastic wrappers and are thus maintained in an environment essentially anaerobic. However, the prevailing record of safety for some of these foods has been based on packaging and marketing in a manner that allows access of air to the surfaces of the foods. It may not be desirable to use this newer method of packaging for processed nonsterile foods of a kind in which micro-organisms can multiply, without first investigating the effect of an anaerobic environment upon the microbial ecology of the foods.

Two organisms are of special interest in this connection, namely *Clostridium botulinum* and *Staphylococcus aureus*. It would seem expedient to determine whether the production of food poisoning toxins may be favoured by the changed environment, and if such toxins may be produced in the absence of ancillary spoilage. Spoilage caused by common aerobic bacteria is often a deterrent to the consumption of foods containing toxins which could render them dangerous. The competitive action of spoilage bacteria under certain conditions may also repress multiplication of specific pathogens.

Two fatal cases of botulism involving smoked fish vacuum packed in plastic wrappers have been reported from the United States of America (Report, 1960). Dr. C. E. Dolman of the University of British Columbia (pers. comm.) isolated *Clostridium botulinum* type E and demonstrated the toxin from this organism from residual fragments of the incriminated samples. Dr. M. T. Bartram of the Food and Drug Administration, Washington (pers. comm.) has informed us that his colleagues also demonstrated the toxin in fragments of the fish. Similar specimens from the same factory were obtained for investigation of the production of toxin under aerobic and anaerobic conditions.

Sliced bacon is also vacuum packed in plastic wrappers by some producers. Specimens have been examined by Eddy & Ingram (1962), and their findings suggest that potentially enterotoxic staphylococci may be able to grow on commercial vacuum packed bacon.

METHODS

Formation of the botulism toxin in smoked fish

Portions of the smoked fish were individually inoculated with 0·1 ml of a suspension of spores of *Cl. botulinum*, types A and E, by smearing drops of the suspensions over the surface of the fish. This gave an inoculum of approximately 4,000 spores/g of fish. Duplicate preparations were incubated aerobically and in a Brewer jar in an atmosphere of hydrogen after catalytic removal of residual oxygen. After incubation for 8 days at 30° saline extracts were prepared from each specimen and injected intraperitoneally into mice. As controls, mice protected by antitoxin against each of the types of *Cl. botulinum* were used.

Production of staphylococcal toxins in bacon

Sliced Canadian back bacon, which normally has a salt content of 3–3·2% (w/w) and a nitrite content ranging from 50 p/m to 80 p/m, was inoculated with two strains of staphylococci (about 10^6/g) originally isolated from foods implicated in food poisoning and known to produce toxins causing emesis in cats. The specific emetic toxins, in their purified state, are nonlytic to erythrocytes of rabbit or sheep and are nondermonecrotic and nonlethal (in the sense of a lethal toxin).

Multiple slices of bacon were incubated at 37° in atmospheres of air, 5% CO_2 in O_2, nitrogen at atmospheric pressure, and *in vacuo* after three times flushing the container with hydrogen. The mixture of CO_2 and O_2 was used because our experience showed this to be a suitable atmosphere for toxin production. Commercial nitrogen is sometimes used in conjunction with plastic wrappers, but because commercial nitrogen often contains small amounts of oxygen we also used vacuum treatment after flushing three times with hydrogen, in order to appraise the effect of the absence of oxygen.

Toxins were assayed from an extract of the bacon prepared by comminuting 30 g with 90 ml of NaCl-KCl-tris buffer (pH 7·5) in a Waring blender. Solid particles of food were removed by centrifugation at 10,000 rev/min for 30 min. The supernatant solution was injected intraperitoneally into cats at the rate of 5 ml/cat. Haemolysis was determined by the tube dilution method using solutions containing the appropriate cations. Preparations injected into cats for control purposes were derived from specimens incubated without inoculation, and from inoculated specimens that had been kept frozen at –10°.

RESULTS

Cl. botulinum *toxin.* Mice injected with preparations from noninoculated fish remained healthy. All unprotected mice receiving preparations from inoculated fish died within 24 hr. All mice receiving antitoxins remained alive. The fish inoculated with spores of type A were offensively spoiled, whether incubated aerobically or anaerobically. Aerobically incubated specimens inoculated with type E spores had an off odour but were much less drastically spoiled than those receiving inocula of type A. The specimens incubated anaerobically with type E spores gave no indication of spoilage odour and would probably have been considered edible by people who enjoy smoked fish.

Staphylococcal enterotoxin. The staphylococcal contents after incubation of the bacon, the cat reactions, and the haemolysin titres using erythrocytes of rabbit and sheep are recorded in Table 1. Staphylococci of each strain multiplied profusely in all specimens incubated at 37°, the greater numbers being reached in the presence of oxygen. The cat reactions were unequivocal, typical timing for the onset of emesis and multiple spasms of vomiting being obtained; the control animals all reacted negatively to injections from the uninoculated and the frozen inoculated preparations. Enterotoxin was produced freely under atmospheres of air, 5% CO_2 in O_2, and nitrogen. Only a small amount of enterotoxin was produced under vacuum

Table 1. *Enterotoxin formation by two strains of staphylococci inoculated on to back bacon incubated in different atmospheric environments*

Date of inoculation	Strain inoculated	Atmosphere†	Incubation temp. (°C)	Haemolysin titre with		Cat reaction for enterotoxin‡	Count of staphylococci (/g)
				Rabbit blood	Sheep blood		
10-2-61	ML-5	Air	37	0	0	3	$5 \cdot 1 \times 10^9$
		CO_2–O_2	37	1:4	1:4	5	$7 \cdot 8 \times 10^9$
		Air	−10	0	0	0	$1 \cdot 8 \times 10^6$
	Tadmore	Air	37	0	0	4	$2 \cdot 5 \times 10^{10}$
		CO_2–O_2	37	trace	1:2	2	$2 \cdot 9 \times 10^{10}$
		Air	−10	0	0	0	$2 \cdot 0 \times 10^6$
	None	Air	37	0	0	0	*
		CO_2–O_2	37	0	0	0	*
		Air	−10	0	0	0	*
17-2-61	ML-5	H_2	37	0	0	1	$5 \cdot 5 \times 10^8$
		CO_2–O_2	37	1:2	0	3	$3 \cdot 2 \times 10^9$
		Air	−10	0	0	0	$1 \cdot 9 \times 10^7$
	Tadmore	H_2	37	0	0	0	$4 \cdot 1 \times 10^9$
		CO_2–O_2	37	1:8	1:8	7	$7 \cdot 2 \times 10^9$
		Air	−10	0	0	0	$3 \cdot 0 \times 10^7$
	None	H_2	37	0	0	0	*
		CO_2–O_2	37	Tr. to 1:2	0	0	*
		Air	−10	0	0	0	*
24-2-61	ML-5	N_2	37	0	0	2	$7 \cdot 6 \times 10^8$
		CO_2–O_2	37	0	0	1	$2 \cdot 36 \times 10^9$
		Air	−10	0	0	0	$2 \cdot 2 \times 10^5$
	Tadmore	N_2	37	0	0	1	$3 \cdot 6 \times 10^9$
		CO_2–O_2	37	0	0	0	$4 \cdot 8 \times 10^6$
		Air	−10	0	0	0	$7 \cdot 4 \times 10^5$
	None	N_2	37	0	0	0	*
		CO_2–O_2	37	0	0	0	*
		Air	−10	0	0	0	*

* No typical staphylococci were found in these samples.

† The CO_2–O_2 mixture contained 5% of CO_2; when H_2 was used the packs were flushed with the gas and then incubated under partial vacuum.

‡ Number of bouts of vomiting by the 2 cats used for testing each preparation.

after flushing with hydrogen, a single cat vomiting once. Some variation in toxin production is noted on different days, but this is not unusual as the level of toxin produced even in proven media tends to be inconstant. Haemolysins were produced only in bacon incubated at 37° in an atmosphere of 5% CO_2 in O_2, but at levels much below their emetic threshold.

Thus enterotoxin can be produced in bacon under aerobic and anaerobic conditions. However, the bacon incubated in air and in CO_2–O_2 was obviously spoiled. The odour was offensive, the exposed lean meat was a greyish-straw colour, and many superficial colonies of diverse micro-organisms were evident on exposed surfaces. But the bacon incubated under nitrogen or under vacuum after flushing with hydrogen, in the opinion of a number of people, looked and smelled normal and would have been acceptable to them as food.

Table 2. *The destruction of staphylococcal enterotoxin in bacon by cooking at 205°*

Condition of bacon	Incubation temp. (°C) before cooking	Cooking period (min)	Haemolysin titre (sheep erythrocytes)	Cat reaction for enterotoxin†	Count of staphylococci (/g)
Inoculated with the Tadmore strain of *Staph. aureus**	37	0	1:4	7	$9·4 \times 10^9$
	−10	0	0	1	$1·1 \times 10^6$
	37	6	0	1	0
	−10	6	0	1	0
	37	12	0	0	0
	−10	12	0	0	0
Uninoculated	37	0	0	1	$3·4 \times 10^7$
	−10	0	0	0	$1·4 \times 10^4$
	37	6	0	1	0
	−10	6	0	0	0
	37	12	0	0	0
	−10	12	0	0	0

* This strain produces a heat resistant toxin.

† The number indicates the number of bouts of vomiting by the 2 cats used for testing each preparation.

The effect of cooking upon the activity of toxin in bacon is shown in Table 2. In order to obtain even exposure to heat, the bacon was grilled in an oven at 205°. The temperature of the bacon reached about 88° as shown by a needle type thermocouple skewered through it. All the staphylococci were destroyed by each cooking treatment. Small amounts of toxin remained after the 'medium' cooking, at which time the rashers of bacon were still somewhat flaccid. No toxin was found after the longer cooking period. The particular bacon (purchased retail) contained indigenous staphylococci, which also produced small amounts of toxin.

DISCUSSION

The findings reported suggest that anaerobic packaging of foods formerly marketed in a manner that provided ready access of air, may increase the hazard of food poisoning by allowing toxin production by both *Cl. botulinum* and staphylococci while minimizing the activities of common spoilage organisms whose offensive changes could give warning of potentially unsafe conditions. This may be particularly pertinent to the production of toxin by *Cl. botulinum* type E, for this organism can elaborate toxin at refrigeration temperatures (Ohye & Scott, 1957). This seems to have applied in the fatal cases referred to. Staphylococcal toxin is not completely destroyed unless the bacon is cooked to a crisp condition.

Further investigation is needed on the microbiology of the vacuum packing for those nonsterile processed foods that can allow development of food poisoning toxins. In the meantime foods so treated should preferably be stored in the frozen state.

REFERENCES

EDDY, B. P. & INGRAM, M. (1962). The occurrence and growth of staphylococci on packed bacon, with special reference to *Staphylococcus aureus*. *J. appl. Bact.* (in press).

OHYE, D. F. & SCOTT, W. J. (1957). Studies on the physiology of *Clostridium botulinum*, Type E. *Aust. J. biol. Sci.* **10**, 85.

REPORT (1960). Morbidity and Mortality Weekly Report, **9** (37), 2.

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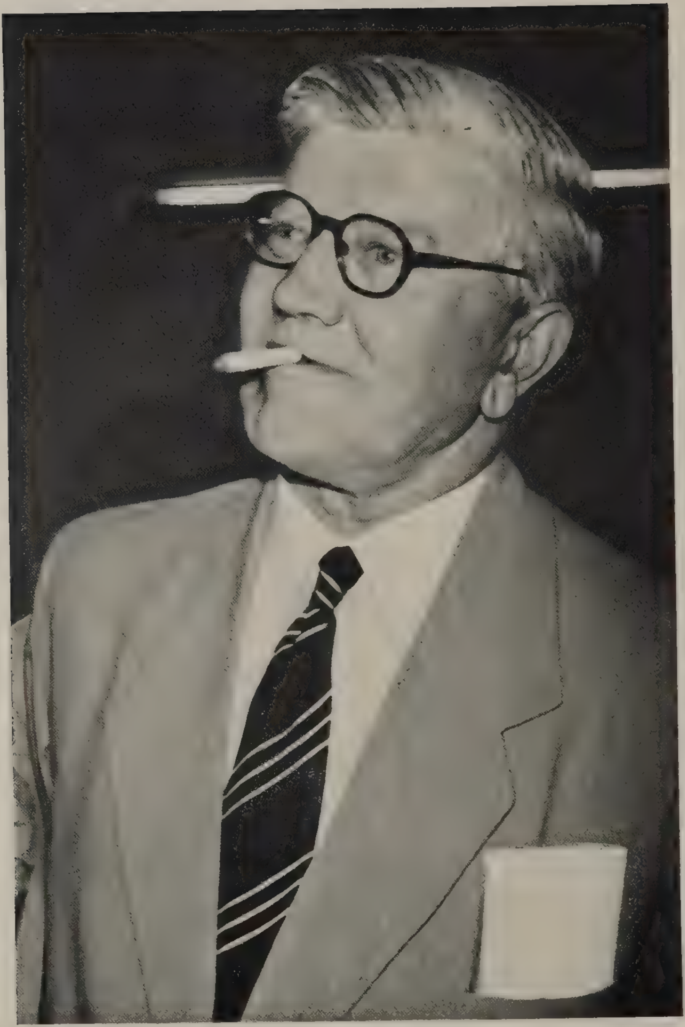

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1894—1962

The death of A. T. R. Mattick marks the end of an era both for the National Institute for Research in Dairying and for our Society. Stenhouse Williams was responsible for the founding of both organizations and Mattick was his chief assistant in both spheres. Today they have achieved a size and standing quite beyond the thoughts of those days.

Mattick was born on September 4th 1894 and educated at Sexe's School, Bruton and University College, Reading, where he took the Diploma in Agriculture and the degree of B.Sc. (Agric.) of the University of London. He joined the staff of the N.I.R.D. in March 1919, when it occupied three rooms in a house in Redlands Road, Reading. Mattick had been engaged in laboratory work in the R.A.M.C. during the 1914–18 war and for the next 10 years Stenhouse Williams, Mattick and W. A. Hoy were to lay the foundations of the science of clean milk production in this country, work in which they received the whole-hearted co-operation of the Ministry's Advisory Bacteriologists. This classical work is still of the greatest value today, and its recommendations are still being applied and its wisdom appreciated.

The writer joined the staff of the Bacteriology Department of the N.I.R.D. in 1927 and naturally came under his wing. As an academic chemist without any experience of bacteriology I had a lot to learn, not only bacteriology but also about milk production and dairying in general. As a 'new boy', my impressions of Mattick at that time were, first, his all-embracing friendliness and desire to give me all the help he could in settling down to my new job, and second, the extraordinary width and depth of his scientific knowledge, which went far beyond the extent of the B.Sc. (Agric.) course. I was particularly impressed by his grasp of the fundamentals of physics and chemistry and greatly benefited from many detailed discussions with him. As an example of his interests I may mention how he, W. J. Wiley (then a visiting worker from Australia) and I used to foregather round his desk at the end of the day's work to study the classical papers of Mansfield Clark on oxidation-reduction potential, then quite an indigestible theme even for physical chemists. Mattick was endowed with great mental and physical energy and inspired us all with his enthusiasm in many directions, both in the Institute and outside it.

His well deserved promotion to Head of the Bacteriology Department came in April 1930, and in the same year he obtained his Ph.D. degree (London). It is characteristic of the man that this work was physical and chemical rather than bacteriological, and Mattick had in fact carried out pioneering work on film formation in milk and on copper-induced oiliness.

About this time he was at the centre of two now famous ventures. The *Journal of Dairy Research* was founded in 1929, and in 1932 Mattick became one of the two editors, serving it in that capacity until 1958. It had become the custom of the Advisory Bacteriologists, teachers of agricultural bacteriology, and others engaged in the dairy industry to come at regular intervals to the N.I.R.D. for discussions, and Stenhouse Williams now called an informal meeting on a rather more comprehensive scale at St. Patrick's Hall, Reading University, for this purpose. This particular conference became the Society of Agricultural Bacteriologists and the six papers given in 1931 were subsequently bound in mimeographed form and so may be regarded as the first volume of our present *Journal*. Over the following 31 years the Society has expanded from the original 33 to nearly 600 members and changed its name, but it is a particular joy to the writer that throughout this time it has retained its character of informality and friendliness, although its size, scope and scientific standing have been enormously enhanced. A large share of the credit for this must go to Mattick, who was its first Secretary and became its second President.

The period from 1927 to 1939 may be regarded as the adolescence of the Bacteriology Department; it slowly passed from the 'clean milk' phase to the highly developed state in which it exists today. Progress was inevitably slow because of inadequate knowledge and severely limited apparatus and financial resources. Special permission had to be obtained to spend a few pounds on new equipment (actually often second-hand) and milk had to be saved up over days, a few gallons at a time, to make cheese experiments. Throughout this period, with the help of Miss E. R. Hiscox, Mattick steered us all with the kindest encouragement and friendliest criticism. His own work was largely on pasteurization, especially the H.T.S.T. process, and contemporary dairy scientists cannot appreciate the difficulties, and especially the hostility from some quarters, which surrounded the subject in those days. The Institute gradually played a more important role in the processing side of the dairy industry and paid attention to such aspects as plant and bottle hygiene, milking machines, the survival of tubercle bacilli, the plate count, methylene blue and other tests for bacteriological quality, the design of pasteurizing and manufacturing plant, and problems in the cheese industry, especially slow starter. The outbreak of the second world war naturally changed the emphasis of Mattick's work. The National Milk Testing and Advisory Scheme was launched and he became the Chairman of the Technical Committee. The Department was also asked to organize a mobile laboratory to be on call to any farm or dairy desiring its help, and in both activities co-operation with the Advisory Bacteriologists was an outstanding feature; between them they, with the industry's bacteriologists, virtually controlled the keeping quality of the nation's milk. These schemes not only maintained bacteriological quality under very difficult circumstances but effected a considerable improvement in the hygienic conditions of cans, pasteurizing plant, tankers and bottles. Other fields of study in which Mattick continually functioned as a leader were: the problems of tests for keeping quality, with Mr. A. Rowlands; phage in starters, with Dr. A. A. Nichols; chemical sterilizers, with Dr. L. F. L. Clegg; the assessment of 'sterility' (or, more accurately, a satisfactory bacteriological condition); and antibiotic production by, and the serology of, lactic streptococci, the last two with Dr. A. Hirsch and Dr. P. M. F. Shattock. Subsequently, fundamental studies of the lactobacilli were started with Dr. M. E. Sharpe, Dr. M. Briggs and Dr. D. M. Wheater, and of the intestinal flora of farm animals with Dr. C. A. E. Briggs. The early work on rennet and other aspects of cheese making and ripening was renewed and taken over by Dr. N. J. Berridge and Dr. L. A. Mabbitt.

In 1946 Mattick was appointed Deputy-Director of the N.I.R.D. and became the second President of the Society of Dairy Technology. He was a founder committee member and associate editor of the Society for General Microbiology, and Chairman of many committees of the Ministry of Agriculture and of the British Standards Institution concerned with dairy bacteriology. Towards the end of his career he received two well deserved honours. He was appointed Acting Director of the N.I.R.D. on the retirement of Professor H. D. Kay in 1958, and awarded the C.B.E. in the 1960 New Year's Honours.

Although he had enjoyed good health during a long and distingiushed career, his health deteriorated rather rapidly during the last few years. Those of us who had been intimately working with him felt that he never fully recovered from the death of his wife in 1943. They were clearly devoted to each other, and in the absence of children this loss had the effect of making him rather withdraw into himself socially. However, he maintained his vigorous interest in all things pertaining to dairy bacteriology in general and to the Institute in particular, up to his retirement in 1959.

Mattick was never afraid to say what he thought, either about things or people. He possessed that supreme courage (which few of us have) of saying things to people's faces and not behind their backs. In spite of this he was never known to lose a friend; the wise appreciate that a candid criticism is more helpful than a hollow flattery. His conversation was brisk, and at times brusque, but a strong sense of humour created an atmosphere that usually smoothed out potential difficulties and in which the wounds made by the arrows that occasionally slipped out and found their mark soon ceased to exist. Until the last few years he was strongly extrovert; in other words he enjoyed life and found great pleasure in mutual enjoyment with his friends. The early days when dairy bacteriologists were few in number, inadequately equipped and financially not so well off as today had their compensations, and those of us who consorted with Mattick in those times will retain many happy memories of those meetings when 20 was a crowd and there was more time for enjoying those activities which have disappeared since the advent of the doubtful advantages of the universal motor car, the aeroplane and the television set. In an age less far removed from the first Elizabethan era when we made our own amusements Mattick was always prominent and his laughter and obvious enjoyment always infectious.

His memory will survive for his friends chiefly as an inspiring example of single-mindedness of purpose and devotion to duty. He lived for the Institute and the cause he served.

This is essentially a personal tribute from one who had the happy privileges of his friendship and of working under him. More detailed information about his scientific work is given in *Dairy Industries* (1955), 415; (1958), 647; the *Journal of the Society of Dairy Technology* (1959), **12**, 232; and *Dairy Industries* (1960), 516, 588.

J. G. Davis

MEANWELL, L. J. (1962). *J. appl. Bact.* **25** (2), 128–136.

THE INFLUENCE OF PENICILLIN ON CHEESE STARTER ACTIVITY

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SUMMARY: The cultivation of cheese starter cultures for 16 hr at 22° in milk containing 0·05 i.u. of penicillin/ml resulted in limitation of cell numbers and consequent loss of activity. Such loss of activity did not occur when heavy inocula and cultivation for 3 hr at 30° were used. The presence of penicillin at a level of 0·05–0·08 i.u./ml in milk for bulk starter cultivated for 16 hr at 22° exerted more influence on subsequent starter activity than was caused by the presence of 0·1 i.u. ml in the activity test milk itself. The cells that survived contact with penicillin appeared to be healthy and capable of normal reproduction.

IT WAS REPORTED by Whitehead & Lane (1956) that whereas in Cheddar cheese manufacture normal cheese was formed despite the presence of 0·05 i.u. of penicillin/ml in the cheese vat milk, 0·1 i.u./ml and over caused the cheese to be pasty and have a yeasty flavour.

Since the use of penicillin for the treatment of bovine mastitis has increased during the past few years, the following more detailed investigation into the influence of penicillin on acid production by cheese starters was undertaken.

METHODS

Propagation of test starter. In the majority of experiments propagation of the test starter was by a 1% (v/v) inoculum into autoclaved milk incubated for 16 hr at 22°. In later tests incubation was for 3 hr at 30° using a 10–15% inoculum. Details of such propagation are given below.

Starter activity. This was measured at 30° in terms of acid production in 10 ml quantities of sterilized milk inoculated with 2% of the test starter. These were titrated with N/9 NaOH and the total acid was calculated as % lactic acid. The original acidity of sterilized milk is 0·19%.

Colony counts. These were made on tryptone-yeast-lactose broth agar plates incubated for 3 days at 30°. The medium contained (all as % w/v): tryptone, 3; yeast extract, 1; lactose, 2; beef extract, 1; K_2HPO_4, 0·5; agar, 2. The medium was adjusted to pH 6·8 and heated at 121° for 5 min. All counts were made either on the experimental milk after receiving a 2% inoculum of the test culture or on such milk during subsequent incubation at 30°.

Milk. The 'penicillin free' milk used was commercially sterilized milk containing less than 0·005 i.u. of penicillin/ml, except for the experiments described in Table 1.

Penicillin. This was added to the milk in the form of 1% (v/v) of an aqueous solution containing an appropriate concentration of the potassium salt of benzyl penicillin.

EXPERIMENTS AND RESULTS

Propagation of starter at 22°

According to Whitehead & Lane (1956), with a healthy culture acid production proceeds normally in cheese milk containing 0·2 i.u. of penicillin/ml until 'whey off'; but there is considerable slowing down of acid production during the subsequent cheddaring process. In the present work this phenomenon was reproduced on a laboratory scale at 30° using a healthy cheese starter and pasteurized milk containing 0·2 i.u. of penicillin/ml. Normal acid production was followed by a slowing down in activity in both liquid milk and in the whey expressed from the curd of a miniature cheese. A typical result of one test is recorded in Table 1.

Table 1. *The effect of penicillin on acid production at* 30° *in liquid milk and in whey in a miniature cheese vat, using a* 2% *inoculum of starter* 1016

Penicillin concn (i.u./ml)	Acid (as % lactic acid) in			
	Milk, after		Whey, after	
	3½ hr	6 hr	4½ hr	6 hr
0·0	0·32	0·60	0·28	0·65
0·2	0·30	0·38	0·26	0·45

In order to study this loss of activity in more detail, hourly estimations were made at 30° of colony counts and acid production by a healthy starter after transfer to milk containing different quantities of penicillin. The results of this test are recorded in Table 2, which shows that with the addition of penicillin at 0·05 i.u./ml to liquid milk acid production proceeded normally for 6 hr, after which there was a slight slowing; bacterial multiplication proceeded at a slower rate than in the penicillin free control, a decrease in count occurring after 9½ hr incubation. These differences were enhanced at higher penicillin concentrations. Considerable slowness in acid production was evident after 4½ hr when the test milk contained 0·1 i.u. of penicillin/ml. After 6 hr the slowness was accompanied by a marked decrease in colony count and after 27 hr the starter organisms had almost completely disappeared. Thus Whitehead's observation on loss of activity appears to be associated with the number of organisms or their rate of multiplication.

Following these experiments at 30°, an examination was made of the effect of the presence of penicillin during propagation on the ability of starters to grow and to form acid in milk. Propagation was for 16 hr at 22°, the usual time-temperature combination used for the preparation of bulk starters for cheese making. Six starters were used. They were grown for a single transfer at 22° in sterilized milk to which had been added 0, 0·05, 0·075 or 0·1 i.u. of penicillin/ml. Each starter was then inoculated (2% v/v) into penicillin free sterilized milk. Colony counts of the milk were made immediately after inoculation and the amount of acid produced (activity) was determined after incubation for 6 hr at 30°. The results of this experiment

Table 2. *The effect of penicillin on the changes in colony count and acidity produced in milk at* 30° *by starter* 924

Time of incubation (hr)	Colony counts (a)* and acidities (b)† with penicillin at (i.u./ml):							
	None		0·05		0·075		0·1	
	a	b	a	b	a	b	a	b
0	23·5	0·19	23·5	0·19	23·5	0·19	23·5	0·19
1	51·0	0·21	49·0	0·20	44·0	0·19	39·0	0·20
2	119	0·21	64·0	0·20	71·0	0·20	57·0	0·20
3	160	0·24	72·0	0·24	105	0·24	66·0	0·24
4½	370	0·33	210	0·33	330	0·33	86·0	0·30
6	490	0·52	225	0·50	170	0·44	49·0	0·36
7½	920	0·70	380	0·65	233	0·61	57·0	0·45
9½	490	0·76	207	0·72	63·0	0·72	24·0	0·56
11½	450	—	161	—	58·0	0·72	5·50	0·60
14	540	—	146	—	41·0	—	1·30	0·71
27	610	—	155	—	36·0	—	0·97	—

* Colony counts in 10^6/ml. † Acidity as % lactic acid.

(Table 3) confirm the findings of Berridge (1956) that there is considerable variation in susceptibility to penicillin from starter to starter. Starter 708 was the least and starter 738 the most susceptible, the rest being intermediate and very similar to one another, as judged by colony count. Even 0·05 i.u. of penicillin/ml of the propagation milk had a marked effect.

Table 3. *The effect of the presence of penicillin during starter propagation* at* 22° *on its colony count and its subsequent ability to form acid in milk at* 30°

Starter no.	Colony counts (a)† and activities (b)‡ with starters grown with penicillin at (i.u./ml):							
	None		0·05		0·075		0·1	
	a	b	a	b	a	b	a	b
708	32·6	0·55	16·2	0·51	17·0	0·53	15·6	0·54
738	16·0	0·48	1·8	0·39	0·1	0·29	0·01	0·20
924	24·4	0·45	13·4	0·40	6·5	0·37	2·3	0·32
990	31·2	0·60	15·3	0·51	8·1	0·47	3·4	0·40
1056	33·2	0·53	17·0	0·44	10·0	0·44	2·4	0·27
1078	34·0	0·75	15·3	0·67	6·8	0·60	2·9	0·45

* Starter propagated for 16 hr at 22°. † Colony count at start, in 10^6/ml. ‡ Activities: as % lactic acid after 6 hr at 30°.

Next, a collection of 27 commercial mixed starters was examined for sensitivity to penicillin during propagation for 16 hr at 22° and for subsequent activity at 30°. Each starter was propagated in milk with and without penicillin and the culture from each propagation was then tested for activity in penicillin free milk and in milk containing 0·1 i.u. of penicillin/ml. Eight of the starters were propagated in milk containing 0·08 i.u. of penicillin/ml and 19 in milk containing 0·05 i.u. of penicillin/ml. Colony counts were made on the penicillin free activity test milks immediately after inoculation, and acid production was measured after incubation for 5–6 hr at 30°.

Table 4. *The effect of the presence of penicillin at 0·08 i.u./ml during commercial starter propagation on its growth and subsequent activity at 30° in milk with and without 0·1 i.u. of penicillin/ml*

Starter no.	Colony counts (a)* and activities (b1, b2)† with starters grown for 16 hr at 22° with penicillin at (i.u./ml):					
	None			0·08		
	a	b1	b2	a	b1	b2
98	6·9	0·44	0·40	0·6	0·29	0·27
99	4·7	0·44	0·41	0·04	0·21	0·21
00	9·2	0·39	0·37	0·3	0·26	0·23
04	6·2	0·41	0·39	<1·0	0·23	0·21
05	8·6	0·36	0·34	0·15	0·23	0·21
06	8·3	0·38	0·35	1·8	0·27	0·24
07	8·7	0·37	0·37	1·3	0·27	0·24
08	7·4	0·42	0·43	1·4	0·28	0·26
Means	7·35	0·404	0·382	0·494	0·255	0·234

* Colony counts: (a), in 10^6/ml, immediately after inoculation.

† Activities: as % lactic acid after 5 hr at 30°, in milk containing no penicillin (b1) and 0·1 i.u. of penicillin/ml (b2).

Table 5. *The effect of the presence of penicillin at 0·05 i.u./ml during commercial starter propagation on its growth and subsequent activity at 30° in milk with and without 0·1 i.u. of penicillin/ml*

Starter no.	Colony counts (a)* and activities (b1, b2)† with starters grown for 15 hr at 22° with penicillin at (i.u./ml):					
	None			0·05		
	a	b1	b2	a	b1	b2
09	11·1	0·59	0·52	1·8	0·45	0·38
10	6·6	0·51	0·56	1·0	0·47	0·40
11	9·1	0·65	0·57	5·1	0·49	0·48
12	6·7	0·62	0·51	2·4	0·48	0·39
13	7·1	0·39	0·34	3·6	0·35	0·31
14	10·6	0·44	0·41	4·4	0·37	0·33
15	8·6	0·47	0·45	6·7	0·41	0·38
16	11·1	0·63	0·57	4·4	0·50	0·40
17	10·3	0·65	0·56	6·5	0·61	0·49
18	3·2	0·71	0·51	2·0	0·64	0·33
19	11·1	0·67	0·56	3·6	0·53	0·47
20	7·4	0·60	0·48	3·2	0·40	0·34
21	5·1	0·58	0·46	1·4	0·40	0·32
22	9·1	0·77	0·60	4·1	0·57	0·50
23	7·6	0·80	0·68	3·6	0·63	0·46
24	6·7	0·70	0·58	3·2	0·57	0·44
27	8·7	0·59	0·53	4·3	0·50	0·42
28	5·5	0·63	0·64	2·0	0·61	0·52
29	7·3	0·62	0·52	3·5	0·45	0·39
Means	8·705	0·615	0·53	3·562	0·495	0·408

* Colony counts: (a), in 10^6 ml, immediately after inoculation.

† Activities: as % lactic acid after 6 hr at 30°, in milk containing no penicillin (b1) and 0·1 i.u. of penicillin/ml (b2).

The results of these tests are recorded in Tables 4 and 5. It is evident that the presence of 0·08 and 0·05 i.u. of penicillin/ml in the milk used for propagation at 22° had more influence on subsequent activities in the penicillin free milk than had the presence of 0·1 i.u. of penicillin/ml in the activity milk on the growth of healthy cultures: i.e., penicillin affects starter activity more if present in bulk starter milk than if present in cheese vat milk.

In order to judge whether cells propagated in milk containing penicillin were healthy the following comparative experiment was made. Two healthy starters were propagated in milk containing 0, 0·05, 0·075 and 0·1 i.u. of penicillin/ml for 16 hr at 22°. Then 2% inocula of these starters were introduced into penicillin free sterilized milk and the inoculated milks were examined immediately for colony count and after 6 hr at 30° for activity. Also, the starters that had been grown in penicillin free milk were inoculated at different rates into penicillin free sterilized milk, and the inoculated milks examined in the same way.

Table 6. *A comparison of the activities of approximately equal inocula of cells of starter cultures grown for* 16 *hr at* 22° *in the presence* (*a*) *and absence* (*b*) *of penicillin*

Starter no.	Code no.	Initial colony count (10^6/ml)		Activity‡ (% lactic acid)	
		a*	b†	a*	b†
924	1	20·4	20·4	0·50	0·50
	2	7·6	5·1	0·45	0·31
	3	2·1	1·0	0·31	0·25
	4	0·27	0·5	0·24	0·24
990	1	16·4	16·4	0·56	0·56
	2	9·7	8·2	0·44	0·37
	3	4·0	4·1	0·32	0·34
	4	0·5	0·8	0·29	0·25

* Penicillin in starter culture: code nos. 1, 2, 3, 4; 0, 0·05, 0·075 and 0·10 i.u./ml, respectively.

† Penicillin free starters, inoculum (% v/v). Starter 924: code nos. 1, 2, 3, 4; 2·0, 0·5, 0·1, 0·05, respectively. Starter 990: code nos. 1, 2, 3, 4; 2·0, 1·0, 0·5, 0·1, respectively.

‡ Activity: in penicillin free milk, 6 hr at 30°.

The results of these tests are recorded in Table 6, from which it appears that, with starters propagated at the same temperature, the activities were very similar no matter whether the cell populations were reduced because of the presence of penicillin during propagation or merely by dilution. It therefore seems that the cells present after growth for 16 hr at 22° in contact with penicillin are healthy cells capable of normal acid production when transferred to a penicillin free medium.

Propagation of starter at 30°

When cultures grown for 16 hr at 22° in penicillin free milk were transferred to milk containing 0·1–0·2 i.u. of penicillin/ml at 30°, acid production appeared to proceed normally for 3 hr (Tables 1 and 2). Also, with culture 924, as compared with penicillin free milk, the proportion of cells present in milk containing 0·1 i.u. of penicillin/ml was greater after 3 hr at 30° (Table 2, 41%) than after 16 hr at 22° (Table 3, 9·4%). Accordingly should it be possible, under normal conditions, to produce almost as many cells by a 3 hr incubation at 30° as are present in a 16 hr 22° culture (this being the usual combination for bulk starter preparation), the number of cells present after contact with 0·1 i.u. of penicillin/ml would be greater after the shorter propagation time.

It was found that in penicillin free milk a colony count of more than half of that given by a 16 hr 22° culture could be obtained by mixing equal quantities of a 16 hr 22° culture and sterilized milk, incubating at 30° for 45 min, transferring a 15% inoculum of this mixture into sterilized milk at 30° and incubating for 3 hr. Without the intermediary 45 min period at 30° the count after the final 3 hr at 30° was too low. With this procedure it was found that it did not matter whether the propagation milk was penicillin free or contained 0·075 i.u. of penicillin/ml, the final colony counts being nearly the same, and in fact a little higher than that given by a 16 hr 22° culture grown in milk containing that concentration of penicillin (see Table 7). Two starters were used, and cultures in penicillin free milk were prepared by the normal method of 16 hr at 22° and by the rapid method at 30°. Parallel cultures were also prepared in milk containing 0·075 i.u. of penicillin/ml. Then all four cultures of each starter were inoculated (2% v/v) into penicillin free milk and into milk containing 0·15 i.u. of penicillin/ml. The initial counts were determined and also the activities after 6 hr at 30°, with the results shown in Table 7. It is clear that when the propagation milk contained 0·075 i.u. of penicillin/ml, starters grown by the rapid method at 30° gave higher colony counts than those grown for 16 hr at 22°. Also their activities relative to those of the starters grown in penicillin free milk were greater than was the case with the 16 hr 22° cultures. For example, with starter 990 and the rapid method at 30°, penicillin in the propagation milk led to a reduction in activity from 0·55 to 0·52, i.e. only 5·4%: but when the same starter was grown for 16 hr at 20° the reduction was from 0·61 to 0·48, or 21·3%. These results were obtained in penicillin free milk, but the situation was very similar when the milk contained 0·15 i.u. of penicillin/ml. Further, starter 924 behaved similarly,

Table 7. *A comparison of the growth and activities of normal starters and those grown quickly at 30° (see text) with an assessment of the effects of penicillin during propagation and in the test milk*

Culture no.	Propagation method			Initial colony count (10^6/ml)	Mean activities† in milk with penicillin	
	Time (hr)	Temp. (°C)	Penicillin		Absent	0·15 i.u./ml
990	16	22	Absent	11·24	0·61	0·50
	16	22	Present*	5·35	0·48	0·38
	3¾	30	Absent	6·38	0·55	0·46
	3¾	30	Present*	6·23	0·52	0·43
924	16	22	Absent	9·84	0·60	0·26
	16	22	Present*	0·27	0·28	0·19
	3¾	30	Absent	6·63	0·51	0·26
	3¾	30	Present*	1·56	0·33	0·20

* Penicillin concentration, 0·075 i.u./ml. † Activity: as % lactic acid after 6 hr at 30°. The values for culture 990 are the means of 5 tests, and those for culture 924 are the means of 4 tests.

though the results were less striking because penicillin in the propagation milk had much greater effects with both methods of propagation. However, in an earlier experiment with culture 924 in which the propagation milk contained 0·05 i.u. of penicillin/ml, the reduction in activity in penicillin free milk was only 2·5% when the culture was grown for 3 hr at 30°, though when it was grown for $4\frac{3}{4}$ hr the reduction was 40%. From these results it appears that it is possible to propagate certain starters in milk containing 0·05–0·075 i.u. of penicillin/ml without serious loss of cell numbers or activity, providing the total time allowed for multiplication of the culture at 30° does not exceed $3\frac{3}{4}$ hr.

Continuous culture of starter

While this investigation was in progress Ashton *et al.* (1959) published details of the continuous culture of cheese starter. In this paper there was no reference to penicillin. After preliminary incubation of milk containing 10% of culture for 3 hr at 30°, milk was allowed to flow continuously into the starter vessel at such a rate that the volume was changed once every hour. Very heavy inocula of this continuous culture starter were used in the cheese vat. A modification of this technique was applied in the present investigation in an experiment in which the initial $3\frac{3}{4}$ hr two stage propagation at 30° was made in penicillin free milk, the inflowing milk contained 0·05 i.u. of penicillin/ml and the rate of flow was such that the volume changed once every 2 hr. Thus the penicillin concentration steadily increased as the continuous culture proceeded. The results are given in Table 8, which shows that whereas in the continuous culture the acidity remained roughly constant for the whole 6 hr period, the colony count (which had fluctuated somewhat during the first 4 hr) suddenly decreased after the 5th hr. The lowered count was accompanied

Table 8. *The development of acidity in a continuous culture* of starter* 924 *at* 30° *and the activities of successive samples in milk with and without penicillin*

Continuous culture*		Initial colony count** (10^6/ml)	Activity† in milk with penicillin	
Time of sampling (hr)	Acidity (% lactic acid)		Absent	Present‡
0	0·46	8·3	0·32	0·31
1	0·44	10·1	0·35	0·32
2	0·46	7·7	0·34	0·30
3	0·50	8·6	0·33	0·30
4	0·46	15·6	0·35	0·34
5	0·47	3·3	0·30	0·23
6	0·44	1·2	—	—

* Milk containing 0·05 i.u. of penicillin/ml flowing into a culture propagated by the rapid process in penicillin free milk. Rate of volume change, once every 2 hr.

** Colony count of milk after adding a 2% inoculation of continuous culture.

† Activity: as % lactic acid, after 5 hr at 30°. ‡ Penicillin concentration: 0·1 i.u./ml.

by a slight loss only of activity in penicillin free milk, but in milk containing 0·1 i.u. of penicillin/ml the loss was heavy.

Thus it appears that from the point of view of combating penicillin contamination of bulk starter supplies, the use of the continuous culture technique will not be successful beyond the 5th hr if the incoming milk supplies are all contaminated with penicillin to the extent of 0·05 i.u./ml.

Discussion

Sometimes penicillin in milk used for the preparation of laboratory cultures and in bulk starter milk is destroyed by the addition of penicillinase, but this practice is by no means general. The addition of penicillinase to cheese vat milk is not considered to be a practicable possibility.

In a large cheese making dairy handling from 30,000 to 50,000 gallons of milk each day, bulked milk has been examined from 1,000 gal vats at frequent intervals and on some occasions a concentration of penicillin of 0·075 i.u./ml has been found. On the other hand in individual farm supplies a concentration of more than 0·2 i.u./ml has frequently been reached. Verlinski (1959) considered that in Israel 20% of all milk supplies contained penicillin residues of 0·2 i.u./ml.

From these figures it is clear that the risk of penicillin affecting the cheese making process is far greater when milk is handled in small volumes than in large, i.e. milk used for bulk starter preparations from ordinary incoming supplies will be more likely to contain a damaging concentration of penicillin than will milk bulked into thousand gallon vats.

Contact with penicillin in milk (0·075 i.u./ml) for 16 hr at 22° has been shown, in the present work, to reduce considerably the number of cells in bulk starter. The number of cells added to the cheese vat controls activity and, with the majority of starter cultures, activity is not seriously affected by 0·1 i.u. of penicillin/ml during the first few hours of contact at cheese making temperature (30°). From this it is seen that the immediate effect of penicillin will be more obvious in the activity of a starter culture propagated in a penicillin contaminated milk supply than in the activity of a healthy culture used in a cheese vat with milk of a similar penicillin content.

The propagation of bulk starter at 30° offers a possible method of overcoming low levels of penicillin contamination. Large scale bulk starter preparation at 30° with an incubation period of only 3 hr would entail frequent inoculation of fresh batches of bulk starter. In order to avoid this frequent inoculation a successful attempt has been made to carry bulk starter by continuous culture technique; but it was found that if the incoming milk all contained 0·05 i.u. of penicillin/ml, within 5 hr the gradual build-up of penicillin reduced the rate of cell multiplication too greatly. However, since in the continuous culture technique of Ashton *et al.* (1959) the milk supply is constantly changing, it is probable that a concentration of 0·05 i.u. of penicillin/ml or damaging concentrations of any other antibiotic would rarely be maintained for more than a few minutes at any one time. Thus this method could be of use in overcoming contamination of bulk starter by antibiotics.

REFERENCES

ASHTON, T. R., AIREY, F. K., LEEDHAM, L. F. & GOMM, R. J. (1959). Continuous cheese starter manufacture. *Int. Dairy Congr., London*, **2**, 605.

BERRIDGE, N. J. (1956). Penicillin in milk. III. The effect of low concentrations of penicillin on the rate of acid production by starter cultures. *J. Dairy Res.* **23**, 348.

VERLINSKI, S. (1959). Penicillin resistant starter. *Int. Dairy Congr., London*, **2**, 594.

WHITEHEAD, H. R. & LANE, D. (1956). The influence of penicillin on the manufacture and ripening of Cheddar cheese. *J. Dairy Res.* **23**, 355.

(*Received* 14 *September*, 1960)

Eddy, B. P. (1962). *J. appl. Bact.* **25** (2), 137–146.

FURTHER STUDIES ON *AEROMONAS*. I. ADDITIONAL STRAINS AND SUPPLEMENTARY BIOCHEMICAL TESTS

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SUMMARY: The results are given of the behaviour of 88 strains of *Aeromonas* in an extended series of biochemical tests. Additional tests confirm the view that *Aer. formicans* merits specific rank. It is proposed that *Aer. liquefaciens* be renamed *Aer. punctata* and that *Aer. formicans* be renamed *Aer. caviae*.

Several developments have taken place in this field since the previous paper (Eddy, 1960) was published. Ewing, Hugh & Johnson (1961) have put forward the view that the genus *Aeromonas* should comprise three species, viz. *Aer. hydrophila* (syn. *Aer. liquefaciens*, *Aer. punctata*), *Aer. salmonicida* and *Aer. shigelloides* (C27 strains; Ferguson & Henderson, 1947; Bader, 1954; Ewing & Johnson, 1960). They regarded *Aer. formicans* as merely a biotype of *Aer. hydrophila* and not meriting specific rank. In this view they were supported by Liu (1961), following work on the antigenic behaviour of unpurified extracellular metabolites, and by Page (1962), who worked on strains derived from ulcerated reptiles. Colwell & Liston (1961), on the other hand, having included *Aeromonas* strains in an Adansonian analysis of pseudomonads, deduced that whereas gas producing aeromonads were most similar to the Enterobacteriaceae, a strain of *Aer. formicans* appeared to be more closely related to the *Pseudomonas* group.

Hugh (pers. comm.) has shown that the anaerogenic, V-P negative *Pseudomonas caviae*, described and named by Scherago (1936, 1937), is in fact an *Aeromonas* by virtue of its fermentative carbohydrate metabolism and Ewing, Hugh & Johnson (1961) showed that *Ps. hirudinis* (Büsing, 1951–2) is also an *Aeromonas*. Schubert (1960, 1961) reported that both motile and nonmotile aeromonads are lipolytic (towards olive oil) and give positive lecithinase and phosphatase reactions. Park (1962) compared a number of polarly flagellated bacteria belonging to the genera *Aeromonas*, *Pseudomonas* and *Comamonas*.

The present paper contains the results obtained in a study of the behaviour of the original strains employed by Eddy (1960), together with additional *Aeromonas* strains, in an extended series of biochemical tests: the nomenclature of the group is also discussed. The taxonomy of *Aeromonas* and its relationship to the C27 strains, as shown by the results of an Adansonian analysis, will be discussed in a further paper.

MATERIALS AND METHODS

The strains used in this investigation comprised: 52 Voges-Proskauer positive strains designated *Aer. liquefaciens* (nos. 16, 17, 95, 96, 98, 112, 114–119, 138, 139, 142–154, 165, 170, 171, 172, 173, 176–178, 181–183, 199, 204–207, 210, 216, 218, 219, 221 and 228–231); 16 anaerogenic, V-P negative strains designated *Aer. formicans* (nos. 18, 155–157, 159, 172, 194–197, 208, 209, 211, 214, 215 and 249); 12 strains of *Aer. salmonicida* (nos. 141, 161–164, 220 and 222–227); 1 strain each of *Photobacterium harveyi* (no. 174) and *Ph. sepiae* (no. 175); and 7 strains which appeared to occupy

Table 1. *Details of the strains used in this study which are additional to those already listed by Eddy* (1960)

Strain no.	Strain name	Designation and source*	
176	*Aeromonas liquefaciens*	CF 15	Mr. R. G. Board[1]
177	*Aer. liquefaciens*	ED 2/2	
178	*Aer. liquefaciens*	ED 12	
181	*Aer. liquefaciens*	Oa 10	Dr. A. J. Holding[1]
182	*Aer. liquefaciens*	Oa 11	
183	*Aer. liquefaciens*	6,1: A-4NEC 505	Dr. N. E. Gibbons[2]
194	*Aer. formicans*	MJT/F7/37	Dr. M. J. Thornley[3]
195	*Aer. formicans*	MJT/F7/39	
196	*Aer. formicans*	MJT/F7/75	
197	*Aer. formicans*	MJT/F7/81	
199	*Aer. liquefaciens*	MJT/F7/50	
202	*Aer. punctata*	NCMB 73	
203	*Aer. punctata*	NCMB 74	
204	*Aer. punctata*	NCMB 77	
205	*Pseudomonas hirudinis*	NCMB 78	(Büsing, 1951–2)
206	*Aer. liquefaciens*	NCMB 79	
207	*Aer. hydrophila*	NCMB 92	(Strain DF2 of Miles & Miles, 1951)
208	*Aeromonas*	1	Dr. W. H. Ewing[4] (Ewing, Hugh & Johnson, 1961)
209	*Aeromonas*	5	
210	*Aeromonas*	11	
211	*Aeromonas*	21	
212	*Aeromonas*	33	
213	*Aeromonas*	34	
214	*Aeromonas*	38	
215	*Aeromonas*	45	
216	*Aeromonas*	46	
217	*Aeromonas*	49	
218	*Aeromonas*	50	
219	*Aeromonas*	54	
220	*Aer. salmonicida*	68	
221	*Aer. liquefaciens*		Dr. K. B. Rogers[5]
222	*Aer. salmonicida*	4617	Dr. Isabel Smith[6]
223	*Aer. salmonicida*	5183	
224	*Aer. salmonicida*	5221	
225	*Aer. salmonicida*	5312	
226	*Aer. salmonicida*	5445	
227	*Aer. salmonicida*	5551	
228	*Aer. liquefaciens*	46	Dr. R. H. Vaughn[7]
229	*Aer. liquefaciens*	160	
230	*Aer. liquefaciens*	163	
231	*Aer. liquefaciens*	175	
249	*Ps. caviae*	NRRL 966	(Scherago, 1936, 1937)

* NCMB, National Collection of Marine Bacteria;
NRRL, Northern Regional Research Laboratory.

[1] The Edinburgh School of Agriculture. [2] National Research Council, Ottawa. [3] Low Temperature Research Station, Cambridge. [4] Communicable Disease Center, Atlanta, Georgia, U.S.A. [5] The Children's Hospital, Birmingham. [6] Scottish Home Dept., Marine Laboratory, Aberdeen. [7] Dept. of Food Science and Technology, University of California.

an intermediate position between *Aer. liquefaciens* and *Aer. formicans* (nos. 113, 140, 202, 203, 212, 213 and 217).

The strains used in this study which are additional to those already listed by Eddy (1960) are given in Table 1. The following strains, excluded from *Aeromonas*, were not used in this further investigation: nos. 97, 108, 158, 160, 166, 167, 168 and 169.

Biochemical tests

The tests used included most of those described previously, with the following additions.

Elastase production. Ability to hydrolyze elastin was demonstrated by clearing of the medium following growth on 1% (w/v) peptone water agar containing 0·5% (w/v) of finely divided elastin prepared by the method of Partridge, Davis & Adair (1955).

Fibrinolysin production. This was determined by observing clearing around the growth on peptone water agar containing 12% (v/v) of citrated human plasma, coagulated by heating the agar at 56° for 15 min before pouring the plates (Christie & Wilson, 1941).

Hydrolysis of coagulated egg albumen and bovine plasma albumen. As above, but using 0·5% (w/v) of heat coagulated and acetone dried egg white or bovine plasma albumen fraction V (Armour Laboratories, Eastbourne).

Lysine and ornithine decarboxylase production. The technique of Møller (1955) was used.

Lecithinase production. This was studied by observing the development of opacity around or below the growth of peptone water agar containing 10% (v/v) of egg yolk emulsion (Oxoid).

Hydrolysis of chitin. Clearing around the growth on a medium containing 0·5% (w/v) of finely divided chitin prepared as described by Pohja (1960) was regarded as evidence of ability to hydrolyze that substance. Strains of *Aeromonas* were shown to hydrolyze chitin by Clarke & Tracey (1956).

Lipolytic activity. The test was to look for the deposition of insoluble oleate round the growth on peptone water agar containing 1% (v/v) of Tween 80 (polyoxyalkylene derivative of sorbitan mono-oleate; Sierra, 1957).

Phosphatase production. The organisms were grown on nutrient digest agar containing 0·01% (w/v) of phenolphthalein phosphate and the cultures tested for the presence of free phenolphthalein by exposure to ammonia vapour (Barber & Kuper, 1951).

Gluconate oxidation (Haynes, 1951). The medium of Shaw & Clarke (1955) was used; after 48 hr incubation the cultures were tested with 'Clinitest' tablets (Ames Co., Nuffield House, London, W.1).

RESULTS

The results given by the new strains (nos. 176–8, 181–3, 186–9, 194–7, 199, 201–247 and 249) in the tests already described (Eddy, 1960) are incorporated in Tables 2–5. The results obtained with all the available strains in the new tests are given below and summarized in Tables 2–5.

Table 2. *The fermentative abilities of* Aeromonas *strains*

Test	Nos. of strains positive, out of		
	51 *Aer. liquefaciens*	16 *Aer. formicans*	12 *Aer. salmonicida*
Gas production*	49	0	12
Fermentation of			
glucose	51	16	12
raffinose	0	0	0
cellobiose	19	13	0
lactose	19	8	0
maltose	51	16	12
sucrose	47	15	0
trehalose	51	16	2
fructose	51	16	12
galactose	51	16	12
mannose	51	12	4
rhamnose	5	0	0
arabinose	36	14	4
xylose	0	0	0
adonitol	0	0	0
dulcitol	0	0	0
inositol	0	0	0
mannitol	51	16	12
sorbitol	22	2	0
glycerol	51	15	12
dextrin	51	16	12
starch	51	15	12
inulin	0	0	0
glycogen	50	15	12
salicin†	31	13	0
salicin‡	39	—	12
Hydrolysis of aesculin§	39	13	12

* Gas production from glucose or, when equivocal, from sodium formate. † Incubated at usual temperature, i.e. 30° (25° for *Aer. salmonicida*). ‡ Incubated at lower temperature, i.e. 25° (15° for *Aer. salmonicida*). § Included in this Table because of its relationship to salicin fermentation.

Table 3. *The proteolytic and related activities of* Aeromonas *strains*

Test	Nos. of strains positive, out of		
	51 *Aer. liquefaciens*	16 *Aer. formicans*	12 *Aer. salmonicida*
Gelatin liquefaction	51	15	12
Hydrolysis of casein	51	16	12
Hydrolysis of coagulated egg albumen	51	15	12
Hydrolysis of coagulated bovine plasma albumen	38	3	0
Fibrinolysis	51	15	12
Elastase	40	2	12
Arginine dihydrolase	51	16	12
Lysine decarboxylase	0	1 (?)	0
Ornithine decarboxylase	0	0	0
Indole production	49	15	0

Elastase production. All 12 strains of *Aer. salmonicida* were able to hydrolyze elastin, as were 40 (78%) of the strains of *Aer. liquefaciens*, though nos. 16, 96, 139, 147, 171, 173, 204–6, 216 and 228 were unable to do so. Only 2 (13%) of the strains of *Aer. formicans* (nos. 214 and 215) and two of the intermediate strains (nos. 140 and 217) had this ability.

Table 4. *Miscellaneous biochemical reactions of* Aeromonas *strains*

Test	Nos. of strains positive, out of		
	51 *Aer. liquefaciens*	16 *Aer. formicans*	12 *Aer. salmonicida*
Oxidase (Kovacs')	51	16	12
Voges-Proskauer	51	0	0
Citrate utilization	40	11	0
Urease	0	0	0
Nitrate reduction	51	16	12
Filtrable haemolysin	44	4	12
Lecithinase	51	15	12
Malonate utilization	0	0	0
Lipase	51	16	12
Black rot in hen eggs	51	12	0
Gluconate oxidation	45	0	0
Hydrolysis of chitin	51	16	0
Cyanide	51	13	0

Fibrinolysin production. All the strains of *Aer. liquefaciens* and *Aer. salmonicida* were positive, and so were all the strains of *Aer. formicans* except no. 249.

Hydrolysis of coagulated egg albumen and bovine plasma albumen. The results for egg albumen were the same as for fibrinolysin, but a degree of differentiation was obtained with the bovine plasma albumen which appeared to be less readily attacked. No strain of *Aer. salmonicida* was positive in this test; 3 (19%) of the strains of *Aer. formicans* (nos. 211, 214 and 215) were positive, as were 38 (75%) of the strains of *Aer. liquefaciens* (nos. 96, 98, 115, 138, 139, 147, 148, 153, 173, 206, 216, 219 and 221 failed). The lower activity of *Aer. formicans* than *Aer. liquefaciens* against coagulated plasma albumen parallels the observation that with *Aer. formicans*, gelatin liquefaction is generally less rapid than with *Aer. liquefaciens*. Two of the *Aer. formicans* strains which hydrolyzed coagulated plasma albumen also hydrolyzed elastin (nos. 214 and 215).

Lysine and ornithine decarboxylase production. Negative results were obtained with all the strains except nos. 196, 212 and 213, which showed weak lysine decarboxylase activity.

Lecithinase production. A positive egg yolk reaction was given by all the strains except nos. 212, 213 and 249.

Hydrolysis of chitin. All the *Aer. salmonicida* strains were negative and all the *Aer. liquefaciens* and *Aer. formicans* strains were positive. Among the positive strains there was great variation in the velocity of the reaction, some being positive after 1 day's incubation but others only after 7–10 days.

Lipolytic activity. When Tween 80 was used as substrate, a positive reaction was given by all the strains.

Table 5. *The biochemical reactions of anomalous* Aeromonas *and* Photobacterium *strains*

Test	Reactions* given by strains nos.						
	113	140	202/203	212/213	217	174	175
Gas production	+	+	+	+	−	−	−
Fermentation of							
glucose	+	+	+	+	+	+	+
raffinose	−	−	−	−	−	−	−
cellobiose	−	+	+	+	−	+	+
lactose	−	−	+	−	−	+	+
maltose	+	+	+	+	−	+	+
sucrose	+	+	−	+	−	−	+
trehalose	+	+	+	+	+	+	+
fructose	+	+	+	+	+	+	+
galactose	+	+	+	+	−	+	−
mannose	+	+	+	+	−	+	+
rhamnose	−	−	−	−	−	−	−
arabinose	+	−	+	−	−	−	−
xylose	−	−	−	−	−	−	−
adonitol	−	−	−	−	−	−	−
dulcitol	−	−	−	−	−	−	−
inositol	−	−	−	−	−	−	−
mannitol	+	+	+	+	−	+	+
sorbitol	−	−	−	−	−	−	−
glycerol	+	+	+	+	−	+	−
dextrin	+	+	+	+	−	+	+
starch	+	+	+	+	−	+	+
inulin	−	−	−	−	−	−	−
glycogen	+	+	+	−	−	+	+
salicin	+	+	+	−	−	+	+
Hydrolysis of aesculin	+	+	+	−	−	+	+
Gelatin liquefaction	+	+	+	+	+	+	+
Casein hydrolysis	+	+	+	+	+	+	+
Hydrolysis of coagulated egg albumen	+	+	+	+	+	+	+
Hydrolysis of coagulated bovine plasma albumen	−	−	−	−	+	−	+
Fibrinolysin	+	+	+	+	+	−	−
Elastase	−	+	−	−	+	−	−
Arginine dihydrolase	+	+	+	+	+	−	−
Lysine decarboxylase	−	−	−	+ (?)	−	+	+
Ornithine decarboxylase	−	−	−	−	−	−	+
Indole production	+	+	−	+	−	+	+
Oxidase (Kovacs')	+	+	+	+	+	+	+
Voges-Proskauer	−	−	−	−	−	−	−
Citrate utilization	−	−	−	+	+	−	−
Urease	−	−	−	−	−	−	−
Nitrate reduction	+	−	+	+	+	+	+
Filtrable haemolysin	+	+	+	+	+	−	+
Lecithinase	+	+	+	−	+	+	+
Malonate utilization	−	−	−	−	−	−	−
Lipase	+	+	+	+	+	+	+
Black rot in hen eggs	−	+	+	+	+	−	−
Gluconate oxidation	−	−	−	−	−	−	−
Hydrolysis of chitin	+	+	+	+	+	+	+
Cyanide	+	+	+	−	+	−	−

* Reactions: + positive; − negative.

Phosphatase production. Most of the *Aer. liquefaciens* and *Aer. formicans* strains gave a pale pink colouration, indicating weak phosphatase activity. The colouration was much less intense than that given by *Staphylococcus aureus*. Some differentiation was noticed in that no colouration was detected in colonies of *Aer. salmonicida* strains (cf. Schubert, 1961). However, the positive reaction was so weak that it is undesirable to attach any significance to it.

Gluconate oxidation. Positive reactions were given by most of the *Aer. liquefaciens* strains (nos. 95, 98, 112, 152, 171 and 173 failed), but by none of the strains belonging to the other groups.

The results obtained in the additional tests further illustrate the unusually comprehensive range of biochemical activities exhibited by members of the *Aeromonas* group. Especially interesting are the hydrolysis of elastin and chitin and the oxidation of gluconate, since these reactions strengthen the division of the group into the three species previously proposed (Eddy, 1960).

In certain respects, and particularly with gas production, the results of the present writer differ from those of Ewing *et al.* (1961) with the same strains. For example, strains 203, 207, 212, 213, 216 and 218 were all found to produce gas from glucose, whereas Ewing *et al.* found none of these able to do so, though nos. 203 and 207 did produce gas from other carbohydrates. Gas production from glucose may be confirmed by demonstrating gas production from sodium formate (by formic hydrogenlyase).

DISCUSSION

Taxonomy of Aeromonas

Aeromonas salmonicida is clearly differentiated from other members of the group, both biochemically and by virtue of its lack of motility, and certainly merits specific rank. The present writer, however, feels that it has a stronger claim to be included in the genus than is conceded by Ewing *et al.* (1961).

It has been suggested (Ewing *et al.* 1961) that the *formicans* strains are merely biotypes of *liquefaciens*, meriting at most subspecific or varietal rank; but this does not seem adequate treatment in the light of the numbers found. Of the 74 *liquefaciens-formicans* strains (i.e. motile aeromonads) investigated 2 were V-P positive and anaerogenic, whereas 6 were V-P negative and aerogenic: hence one would be led to expect only $\frac{2\times 6}{74^2}$ V-P negative, anaerogenic strains. This is a very much smaller number than was actually encountered (16), and if one were also to take into consideration gluconate oxidation the number of V-P negative, anaerogenic and gluconate negative strains expected would be vanishingly small. However, Page (1962) also questioned the subdivision into two species, his arguments being that very weak acetoin producers might give falsely negative V-P reactions if tested by an insensitive qualitative method; that intermediate strains occur (aerogenic, V-P negative and anaerogenic, V-P positive); and that the two types could not be separated by pathogenicity tests. For reasons given elsewhere (Eddy, 1961), quantitative differences in the amount of acetoin found in a culture are of little significance and the V-P test employed is sensitive to less than 1 p/m. The present writer's pathogenicity experiments with goldfish suggested that *liquefaciens* strains were more virulent than *formicans* strains.

Liu (1961), on the basis of cross reactions obtained between two strains of *Aer. formicans* and an antiserum prepared against the (unpurified) extracellular metabolites of a strain of *Aer. liquefaciens*, considered that *formicans* is a mere biochemical variant of *liquefaciens*. Despite the many similarities between these two species it is the opinion of the present writer that this view cannot be sustained. It seems that, although the absence of cross reactions in Liu's technique means that species are different (e.g. *liquefaciens* and *salmonicida*), the presence of cross reactions does not necessarily mean that they are the same.

The division of the motile aeromonads into two species, one V-P positive and aerogenic and the other V-P negative and anaerogenic, is strengthened by the ability of most of the former but none of the latter to oxidize gluconate and by the rather weaker proteolytic powers of the latter. Of a total of 74 such strains studied, 67 may be assigned to either *liquefaciens* or *formicans* on this basis, though the former includes two strains which failed to produce gas though they oxidized gluconate (nos. 210 and 219). There remain 6 aerogenic V-P negative strains (4 of which constitute two identical pairs), all of which were gluconate negative and 1 anaerogenic, V-P negative strain which fermented only glucose, fructose and trehalose (Table 5). These strains cannot with certainty be assigned to either *liquefaciens* or *formicans* and are probably best described as intermediate or irregular. The existence of such anomalous strains is hardly surprising; analogous Enterobacteriaceae are frequently encountered.

Further consideration of the strains of *Photobacterium harveyi* and *Ph. sepiae* (nos. 174 and 175 respectively) has somewhat reduced their claim to be included in *Aer. formicans*, because they both showed lysine decarboxylase activity and the latter ornithine decarboxylase activity also. There seems little justification for regarding these strains as distinct species.

Nomenclature

Since the previous paper (Eddy, 1960) was published, the writer has been stimulated by the interest of Dr. R. E. Buchanan and by the paper of Ewing *et al.* (1961) to a careful appraisal of the nomenclature of the motile aeromonads. This has led to the following proposals.

1. *Aeromonas liquefaciens.* The specific epithet *liquefaciens* was published by Beijerinck (1900) for an organism described by him and unmistakably an aeromonad: its gas production precludes its being *Vibrio*. The epithet *hydrophila* derives from a renaming by Chester (1901) of *B. hydrophilus fuscus* (an illegitimate trinomial), inadequately described by Sanarelli (1891); similar organisms were shown by Russell (1898) to be polarly flagellated. The epithet *punctata* is taken from an organism inadequately described by Zimmerman (1890). In 1896 Lehmann & Neumann, who, to judge from remarks in their preface, must have had access to Zimmerman's strain, published under the same name a description which is unmistakably that of an *Aeromonas*. It is proposed therefore that the correct name for the type species of the genus should be *Aeromonas punctata* (Zimmerman, 1890, *sensu* Lehmann & Neumann, 1896) Snieszko, *comb. nov.*

2. *Aeromonas formicans.* Hugh (pers. comm.) has recently pointed out that *Pseudomonas caviae* (Scherago, 1936, 1937) is, in fact, an aeromonad. A study of Scherago's culture has confirmed this: it proved somewhat atypical in that it was only weakly proteolytic, showed no lecithinase activity and did not produce a black rot in hen eggs. Since the isolation and description of *Ps. caviae* and the valid

publication of the name antedate those of *Aer. formicans* (Crawford, 1954), we believe that the correct name for this species should be *Aer. caviae* (Scherago) *comb. nov.*

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REFERENCES

BADER, R. E. (1954). Ueber die Herstellung eines agglutinierenden Serums gegen die Rundform von *Shigella sonnei* mit einem Stamm der Gattung *Pseudomonas*. *Z. Hyg. InfektKr.* **140**, 450.

BARBER, M. & KUPER, S. W. A. (1951). Identification of *Staphylococcus pyogenes* by the phosphatase reaction. *J. Path. Bact.* **63**, 65.

BEIJERINCK, M. W. (1900). Schwefelwasserstoffbildung in den Stadtgräben und Aufstellung der Gattung *Aerobacter*. *Zbl. Bakt.* (Abt. 2) **6**, 193.

BÜSING, K.-H. (1951–2). *Pseudomonas hirudinis*, ein bakterieller Darmsymbiont des Blutegels (*Hirudo officinalis*). *Zbl. Bakt.* (Abt. 1 Orig.) **157**, 478.

CHESTER, F. D. (1901). *A Manual of Determinative Bacteriology*, p. 235. New York: The Macmillan Co.

CHRISTIE, R. & WILSON, H. (1941). A test of staphylococcal fibrinolysis. *Aust. J. exp. Biol. med. Sci.* **19**, 329.

CLARKE, P. H. & TRACEY, M. V. (1956). The occurrence of chitinase in some bacteria. *J. gen. Microbiol.* **14**, 188.

COLWELL, R. R. & LISTON, J. (1961). Taxonomic relationships among the pseudomonads. *J. Bact.* **82**, 1.

CRAWFORD, I. P. (1954). A new fermentative pseudomonad, *Pseudomonas formicans* n. sp. *J. Bact.* **68**, 734.

EDDY, B. P. (1960). Cephalotrichous, fermentative Gram-negative bacteria: the genus *Aeromonas*. *J. appl. Bact.* **23**, 216.

EDDY, B. P. (1961). The Voges-Proskauer reaction and its significance: a review. *J. appl. Bact.* **24**, 27.

EWING, W. H. & JOHNSON, J. G. (1960). The differentiation of *Aeromonas* and C27 cultures from Enterobacteriaceae. *Int. Bull. bact. Nomen. Taxon.* **10**, 223.

EWING, W. H., HUGH, R. & JOHNSON, J. G. (1961). *Studies on the* Aeromonas *group*. U.S. Dept. of Health, Education and Welfare, Communicable Disease Center, Atlanta, Georgia, U.S.A.

FERGUSON, W. W. & HENDERSON, N. D. (1947). Description of strain C27: a motile organism with the major antigen of *Shigella sonnei* phase 1. *J. Bact.* **54**, 179.

HAYNES, W. C. (1951). *Pseudomonas aeruginosa*—its characterization and identification. *J. gen. Microbiol.* **5**, 939.

LEHMANN, K. B. & NEUMANN, R. (1896). *Atlas und Grundriss der Bakteriologie und Lehrbuch der speciellen bakteriologischen Diagnostik*, vol. 2, p. 240. München: J. F. Lehmann.

LIU, P. V. (1961). Observations on the specificities of extracellular antigens of the genera *Aeromonas* and *Serratia*. *J. gen. Microbiol.* **24**, 145.

MILES, E. M. & MILES, A. A. (1951). The identity of *Proteus hydrophilus* Bergey *et al.* and *Proteus melanovogenes* Miles & Halnan, and their relation to the genus *Aeromonas* Kluyver & van Niel. *J. gen. Microbiol.* **5**, 298.

MØLLER, V. (1955). Simplified tests for some amino acid decarboxylases and for the arginine dihydrolase system. *Acta path. microbiol. scand.* **34**, 115.

PAGE, L. A. (1962). Acetylmethylcarbinol production and classification of 37 cultures of *Aeromonas*. *Bact. Proc.* p. 50.

PARK, R. W. A. (1962). A study of certain heterotrophic polarly flagellate water bacteria: *Aeromonas*, *Pseudomonas* and *Comamonas*. *J. gen. Microbiol.* **27**, 121.

PARTRIDGE, S. M., DAVIS, H. F. & ADAIR, G. S. (1955). The chemistry of connective tissues. 2. Soluble proteins derived from partial hydrolysis of elastin. *Biochem. J.* **61**, 11.

POHJA, M. S. (1960). Micrococci in fermented meat products. Thesis: University of Helsinki.

RUSSELL, F. H. (1898). An epidemic septicemic disease among frogs due to the *Bacillus hydrophilus fuscus*. *J. Amer. med. Ass.* **30**, 1442.

SANARELLI, G. (1891). Ueber einen Mikro-organismus des Wassers, welches für Thiere mit veränlicher und constanter Temperatur pathogen ist. *Zbl. Bakt.* **9**, 193, 222.

SCHERAGO, M. (1936). An epizootic septicemia of young guinea pigs caused by *Pseudomonas caviae* n. sp. *J. Bact.* **31**, 83.

SCHERAGO, M. (1937). An epizootic septicemia of young guinea pigs caused by *Pseudomonas caviae* n. sp. *J. infect. Dis.* **60**, 245.

SCHUBERT, R. H. W. (1960). Untersuchungen über die Merkmale der gattung *Aeromonas*. *Zbl. Bakt.* (Abt. 1 Orig.) **180**, 311.

SCHUBERT, R. H. W. (1961). Über die biochemischen Merkmale von *Aeromonas salmonicida*. *Zbl. Bakt.* (Abt. 1 Orig.) **183**, 485.

SHAW, C. & CLARKE, P. H. (1955). Biochemical classification of *Proteus* and Providence cultures. *J. gen. Microbiol.* **13**, 155.

SIERRA, G. (1957). A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substances. *Leeuwenhoek ned. Tijdschr.* **23**, 15.

ZIMMERMAN, O. E. R. (1890). Die Bakterien unserer Trink- und Nutzwässer, insbesondere des Wassers der Chemnitzer Wasserleitung. I. Reihe. *Ber. naturw. Ges. Chemnitz.* **11**, 53.

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Colwell, R. R. (1962). *J. appl. Bact.* **25** (2), 147–158.

THE BACTERIAL FLORA OF PUGET SOUND FISH

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SUMMARY: Several species of marine fish were sampled bacteriologically to determine the bacterial types associated with the skin, gills, gut and mouth of the animals. Analysis of the total sample distribution of 311 pure cultures showed that *Pseudomonas* species composed 44–48% of the floras of all the areas except the mouth, where they comprised 68% of the flora, and *Achromobacter* and *Micrococcus* made up about 17% and 6%, respectively, of the floras from the several sites. A pleomorphic, coccoid, fermentative *Vibrio* type was found in greatest numbers in the gut, although it was present on the gills and, less frequently, on the skin. The biochemical and physiological characteristics of the organisms isolated suggest that detectable differences exist among the bacterial populations from the skin, gills, gut and mouth. Fish caught in the water of Puget Sound by hand line, deep water otter trawl, inshore otter trawl, beach seine and purse seine were examined bacteriologically to determine the effects of catching method on the qualitative distribution of types, and 299 pure cultures were isolated and extensively tested. The hand line samples yielded a flora consisting predominantly of *Pseudomonas* and *Achromobacter*, though *Micrococcus* and *Flavobacterium* were present in significant numbers. Some differences were observed between the bacterial populations of the fish captured by the other methods, particularly in the presence of *Bacillus* and *Corynebacterium* and of miscellaneous types. These differences, in general, could be attributed to the catching operation.

An extensive literature exists concerning studies of the bacterial flora of marine fish. The earlier investigations were carried out to determine the incidence of enterobacterial types (Gibbons, 1934*a*,*b*; Griffiths, 1937) and to resolve the question of the sterility of fish flesh (Proctor & Nickerson, 1935; Bedford, 1937). The emphasis in most of the studies was, however, on those micro-organisms associated with spoilage reviewed by Griffiths (1937), Reay & Shewan (1949) and Tarr (1954). In addition, there are a number of well documented papers dealing with the natural flora of marine fish (Stewart, 1932; Shewan, 1938; Thjøtte & Sømme, 1943; Aschehoug & Vesterhus, 1943; Liston, 1957; Georgala, 1958). In broad summary, the results of these investigations show that members of the genera *Pseudomonas*, *Achromobacter*, *Flavobacterium* and *Micrococcus* predominate and that representatives of the genera *Proteus*, *Sarcina*, *Bacillus*, *Corynebacterium*, *Serratia* and *Vibrio* are encountered frequently in the bacterial flora of marine fish in the natural environment.

Harrison (1929), Gibbons (1934*a*,*b*) and Blake (1935) have discussed the question of whether there is a commensal flora in the gut of marine fish comparable to that of warm-blooded mammals. They stressed the importance of topographical factors, which might influence the type of feeding and the presence or absence of pollution, in determining the intestinal flora of fishes. Furthermore, there is strong evidence that the bacterial content of the gut depends on whether or not the fish are feeding (Obst, 1919; Hunter, 1922*a*,*b*; Aschehoug & Vesterhus, 1943; Margolis, 1953). The flora of haddock intestine was studied by Gee (1927, 1930), Reed & Spence (1929) and Stewart (1932). The results of these investigations are somewhat conflicting. Gee (1927, 1930) reported the presence of many *Bacillus* species in the intestine, and Reed & Spence (1929) found large numbers of *Proteus* there, whereas Stewart (1932) observed a flora of *Achromobacter*, *Flavobacterium* and some sporeformers. The occurrence of luminous bacteria in the intestine of marine fish has been reported by several investigators (Stewart, 1932; Liston, 1954; Spencer, 1961) and it has been suggested that this type of micro-organism may constitute a portion of the gut commensal flora. Anaerobes have been isolated from fish, particularly from the intestine (Shewan, 1938; Reay & Shewan, 1949), but they do not appear to constitute a significant portion of the normal bacterial flora.

Liston (1957) reported that the bacterial populations of the skin and gills of North Sea skate and lemon sole were similar in both fish and were composed principally of Gram-negative rods of the genera *Pseudomonas* and *Achromobacter*. A marked difference was noted between the bacterial floras of the gut and those of skin and gills, however.

The slime, i.e. the skin, flora has been extensively studied for a number of species of fish from widely different geographical locations (Sanborn, 1930, 1932; Nair, 1934; Kiser, 1944; Kiser & Beckwith, 1942, 1944; Thjøtte & Sømme, 1943; Wood, 1940; Venkataraman & Sreenivasan, 1952, 1954; Dyer, 1947; Liston, 1956, 1957; Georgala, 1958). The general conclusion would seem to be that representatives of the taxonomic groups normally regarded as autochthonous to the soil, air and sea water of the marine environment (ZoBell, 1946) constitute the bulk of the natural flora of the slime on marine fish: they belong chiefly to the genera *Pseudomonas*, *Achromobacter* and *Flavobacterium*.

A factor which must be considered as a source of variation in the natural flora of marine animals is the method of capture. Netting or trawling was used in most of the reported investigations, though Bedford (1937) did study line caught halibut, obtaining results which showed that line caught fish yielded bacterial floras ". . . most like that of fish in the natural environment". Shewan (1949) showed that trawled fish usually carry bacterial loads 10 to 100 times heavier than lined fish and attributed this increase to dragging along the sea floor during the trawling process itself, as well as to handling effects. Comparisons of the occurrence and distribution of bacterial types on fish caught by various methods have not been reported.

Very few studies of the natural flora of fish in the Puget Sound region of Washington exist and these are, more or less, surveys based on incomplete classifications of the bacterial types observed (Nair, 1934; Snow & Beard, 1939). In the course of an investigation of the commensal flora of marine animals (Colwell, 1961), it was desirable to know what variations could be expected in the bacterial populations associated with marine fish captured by various methods. In addition, since it was felt that the several sites on the animal body, such as the skin, gills, gut and mouth, should offer unique micro-environments, the bacterial floras of these areas were examined. In this paper, the results obtained from the investigations are described.

MATERIALS AND METHODS

Species and sources of fish examined. The fish used in the studies were caught during the course of a more general ecological study of the marine fishes of Puget Sound (Logbook, M.V. Commando, College of Fisheries Research Vessel, University of Washington). The area of Puget Sound in which the studies were carried out lies near Seattle, Washington, U.S.A. Catches for which data are reported in the analyses by method of capture were made during the months December through April, 1959–1960. In addition, data obtained from otter trawling operations during the winter of 1958 are included in the commensal flora analyses. Only the predominating animal species in each catch were examined bacteriologically since the several capturing methods, by definition, sample biotopes with a variation in animal species composition (Clemens & Wilby, 1946; DeLacey, unpublished results).

Three hauls, at a depth of 10–13 fathoms and of 10 min duration, were made by otter trawl off Port Orchard on two separate occasions, each of the hauls netting *c.* 70 fish. The species examined bacteriologically included: *Squalus acanthias* Linnaeus (dogfish); *Gadus macrocephalus* Tilesius (cod); *Parophrys vetulus* Girard (English sole); *Merluccius productus* Ayres (Pacific hake); and *Sebastodes melanops* Girard (black rockfish).

Only a few fish were caught by hand line, these catches being made between draggings during the otter trawling operations off Port Orchard. The species sampled were *Squalus acanthias* Linnaeus and *Gadus macrocephalus* Tilesius.

Inshore otter trawling was done in three hauls in Shilshole Bay, directly offshore from Golden Gardens in Seattle. The operation was identical to the deeper water trawling in Port Orchard, but each haul yielded *c.* 30 fish and the fish examined bacteriologically were *Cymatogaster aggregata* Gibbons (ocean perch) and *Ophiodon elongatus* Girard (lingcod).

Platichthys stellatus (Pallas) (starry flounder), *Embiotoca lateralis* Agassiz (sea perch) and *Scorpaenichthys marmoratus* (Ayres) (sculpin) were sampled during a beach seining operation at Golden Gardens beach, which lies within the Seattle city limits, just south of Meadow Point. A net 120 ft long was used and the haul was made during daylight hours (DeLacy & English, 1954), yielding *c.* 25 fish. Since both the inshore otter trawling and the beach seining were not routine operations only a few samplings could be made from these types of catch.

The purse seining was done at Gig Harbour, in three hauls in which *c.* 60 fish in all were taken. The fish sampled bacteriologically were *Oncorhynchus tshawytscha* (Walbaum) (king salmon), *O. kisutch* (Walbaum) (silver salmon), *O. keta* (Walbaum) (chum salmon), *Salmo gairdneri* Richardson (steelhead) and *Clupea pallasi* Valenciennes (Pacific herring).

Collection and examination of fish specimens. Bacteriological samples were taken by the sterile swab technique, using cotton swabs sterilized separately in 16 × 150 mm screwcapped culture tubes containing 1·2 ml of sea water plus 0·5% (w/v) of peptone. The sea water was previously aged by storing it in a glass container in the dark for several weeks to eliminate the intrinsic bacteriostatic property of raw sea water (ZoBell, 1946).

As soon as the fish were taken from the water, an area of skin (*c.* 10 cm^2) from the untouched side of each fish and just below the first dorsal fin at the lateral line was vigorously swabbed. The end of the swab which had been handled during the sampling was broken off and the swab returned to its sterile container. Contact by the fish with the deck, rigging, etc., was avoided as far as possible until the swabbings had been made.

In the cases where the fish were sampled for gill, gut and mouth flora, additional swabs were applied as follows. The aperculum was lifted and a sterile swab inserted into the gill area for swabbing. The gut was sampled by anal insertion with a sterile swab and the mouth was held open at the jaws, allowing vigorous swabbing of the oral tissue.

The culture tubes were brought to the laboratory within 2–4 hr of swabbing and the swabs were streaked on plates of a medium containing: nutrient broth (Difco), 8 g: yeast extract (Difco), 5 g; agar (Difco), 15 g; aged sea water, 1 litre (MacLeod, Onofrey & Norris, 1954). On occasions when the more extensive trawling operations were done, the swabs were stored overnight at 5–8° (refrigerator temperature) prior to streaking on plates.

The plates were incubated for 5 days at 20° (room temperature), when the colonies were picked off into tubes of sea water broth (Difco nutrient broth made up with aged sea water instead of distilled water). An attempt was made in the selection of colonies to ensure that the preponderant types were fairly represented numerically among the isolates, according to their occurrence on the plates. Also, the colonies of unusual appearance (e.g. agar-digesting types) but which did not represent any significantly large part of the flora were chosen, to allow some estimate of the range of bacterial types occurring on the fish. Each isolate was restreaked three times to ensure purity before examination.

Identification of bacteria. The examination comprised determination of morphology (phase contrast, Gram stain, flagella stain), growth characteristics on agar and in broth at 0°, 25°, 37° and 42°, and physiological characteristics, including sea water requirement, presence of catalase and sensitivity to a number of antibiotics (Shewan, Hodgkiss & Liston, 1954). The biochemical tests included gelatin liquefaction, action in litmus milk, nitrate reduction (Greiss-Ilosvay method, Wilson & Miles, 1955), ammonia production in peptone water, growth in Koser's citrate medium, urease production on Christensen's urea agar slants, hydrogen sulphide production from cysteine, acid production from glucose, lactose, sucrose, maltose, mannitol and galactose and hydrolysis of starch. The Voges-Proskauer, methyl red and indole tests were also carried out (Manual, 1957). In addition, more recent and specific procedures such as the tests for oxidase and the oxidation of gluconate to 2-ketogluconate (Kovacs, 1956; Gaby & Free, 1958), the test for oxidative versus fermentative dissimilation of sugars (Hugh & Leifson, 1953) and the trimethylamine oxide reduction test (Wood & Baird, 1943) were done. For further details of the tests used and the procedure followed in the identification of the micro-organisms see Colwell & Liston (1961). All tests were conducted under aerobic conditions and no studies were made of the antigenic structures or serological relationships of the bacteria isolated.

RESULTS AND DISCUSSION

The bacterial floras of skin, gills, gut and mouth

The bacteria isolated from all species of fish captured by deep water otter trawl and hand line in Port Orchard, Washington, and by purse seine in Gig Harbour, Washington, were mainly Gram-negative, asporogenous, rod-like bacilli of the genera *Pseudomonas*, *Achromobacter*, *Flavobacterium* and *Vibrio*. No marked difference between the floras of the fish from the two areas was noted (Colwell, 1961) and the combined data are presented in Table 1. In all, 311 pure cultures were obtained and studied in these analyses.

Table 1. *The percentage incidence of different groups of bacteria* on the skin, gill, gut and mouth of marine fish caught in Puget Sound*

Site of isolation	No. of isolates examined	Percentages of isolates placed in								
		Pseudomonas	Gut group vibrio	*Achromobacter*	*Flavobacterium*	*Corynebacterium*	*Bacillus*	*Micrococcus*	Enterobacteria†	Others‡
Skin	116	43·9	3·4	18·1	8·6	7·8	0·9	6·9	5·2	5·2
Gills	94	44·7	9·5	16·9	7·4	4·2	0·0	8·4	1·5	7·4
Gut	79	48·1	13·9	17·7	2·5	1·3	5·1	5·1	2·5	8·8
Mouth	22	68·3	0·0	18·2	0·0	4·5	0·0	4·5	4·5	0·0

* All species combined and irrespective of method or place of capture.

† *Aerobacter*, *Aeromonas*, *Proteus*.

‡ *Alcaligenes*, *Brevibacterium*, *Arthrobacter*, *Photobacterium*, yeasts.

A major consideration in defining a commensal bacteria flora is the selective action of the site of growth on the animal body. The mammalian gut affords an excellent example of a specific commensal flora, one which has been recognized by bacteriologists for many years, and a similar association of bacterial types with the skin, gills, or gut of marine animals may well exist. The distribution of the types found on the skin and gills and in the gut and mouth of marine fish (Table 1) indicates that the numbers of pseudmonads are roughly the same on all parts of the animal body (*c.* 44–48%), except that somewhat greater numbers were found in samples taken from the mouth (68%). Achromobacters were even more uniformly distributed in the body areas sampled. Similarly, about 6% of the cultures isolated from samples from all the sites were *Micrococcus* species.

The pleomorphic, coccoid, fermentative type of *Vibrio*, the gut group vibrio, was found predominantly in the gut of the animals sampled, but was also obtained from the gills and, in low numbers, from the skin. This finding is similar to those for North Sea skate and lemon sole (Liston, 1957). *Bacillus* species were also found associated with the gut and there was a higher incidence of representatives of *Corynebacterium* on the skin of the fish sampled. It is clear, however, that detailed statistical analysis involving much larger samples would be necessary to discount the effects of sampling error and of other factors which might influence the results.

Table 2 gives some of the results of the biochemical and physiological tests carried out with the pure cultures. Highly oxidative motile rods growing at 0°, insensitive to penicillin, liquefying gelatin and inactive in most carbohydrate test media comprised the bulk of the bacterial populations on the fish sampled. Relatively few of the bacteria isolated from North Pacific fish could grow at temperatures above 30° (Liston & Colwell, 1962) and this was largely true of the Puget Sound isolates. The biochemical data permit some interesting conclusions to be drawn about the different capabilities of the floras of the skin, gills, gut and mouth. Fermentative bacteria were more common in gut and gill samples, undoubtedly because of the incidence of gut group vibrios in these sites. The ability to utilize sugars aerobically was high among the bacteria from all sites. It was noteworthy that starch hydrolysis was most common among the bacteria isolated from the gills; the occurrence of polysaccharide splitting bacteria on the gills of fish has been previously reported by Liston (1957). It is possible that the greater content of mucoid slime in the gills serves as selective agent for polysaccharide utilizing bacteria.

Table 2. *The percentage incidence of certain biochemical and physiological characteristics of bacteria isolated from various sites on marine fish*

Character	Incidence (%) of positive reactions among strains from			
	Skin	Gills	Gut	Mouth
Motility	45·5	54·7	65·0	41·2
Growth at 0°	70·5	81·5	86·5	NT†
Growth at 37°	38·7	46·4	30·2	NT
Sensitivity to penicillin	30·4	33·8	18·4	20·0
Peptonization of litmus milk	29·4	25·0	34·6	54·5
Liquefaction of gelatin	46·8	40·8	47·9	77·3
Production of urease	43·5	36·4	44·6	57·2
Reduction of nitrate	44·2	39·6	48·7	27·3
Reduction of TMO‡	18·5	16·9	15·0	28·6
Hydrolysis of starch	11·2	89·8	21·9	9·1
Production of indole	4·4	0·0	7·1	0·0
Voges-Proskauer reaction	11·0	13·9	16·9	27·3
Methyl red test	3·0	4·5	8·5	0·0
Production of H_2S	12·4	8·2	13·1	18·2
Aerobic Hugh & Leifson test	74·1	75·0	80·8	90·9
Anaerobic Hugh & Leifson test	14·7	27·2	26·3	4·8
Acid from lactose	15·0	15·0	13·9	13·6
Acid from maltose	18·8	27·5	20·2	18·2
Acid from mannitol	15·0	11·4	13·1	13·6
Acid from sucrose	23·6	13·9	17·4	13·6
Acid from galactose	23·3	9·6	6·9	42·9
Growth in Koser's citrate medium	47·7	33·4	4·4	95·4
Production of NH_3 in peptone water	58·8	66·0	14·8	31·8
Catalase	90·6	100·0	89·6	NT
Kovacs oxidase	67·8	61·0	58·6	NT
Cytochrome oxidase	16·1	16·7	20·7	NT
Oxidation of gluconate	0·0	11·8	23·4	NT

† N.T., not tested. ‡ TMO, trimethylamine oxide.

On the basis that growth in Koser's citrate medium can be considered an indication of the ability of micro-organisms to synthesize most of their requirements, it appears that whereas the mouth flora contains a high proportion of nonexacting bacteria the gut flora is composed, to a large extent, of more exacting organisms.

The results of this study, therefore, tend to support the hypothesis that the skin, gills, gut and mouth of marine fish offer specific types of habitat, or niches, for the growth of commensal bacteria, although the differences often may be subtle.

The effect of method of catch on the bacterial flora

In all, 299 pure cultures of bacteria were examined. Preliminary studies (Colwell, 1961; Liston & Colwell, 1962) having demonstrated that the normal bacterial flora of the fish tested showed no host specificity, it has been assumed that the differences in the composition of the flora will represent variations due to the method of capture or to regional differences within Puget Sound. Accordingly the data from the different species have been combined and the distribution in genus groups according to method of capture is given in Table 3.

Table 3. *The percentage incidence of different groups of bacteria in the surface flora of variously caught marine fish**

Method of catching and location	No. of isolates examined	Percentages of isolates placed in								
		Pseudomonas	Gut group vibrio	*Achromobacter*	*Flavobacterium*	*Corynebacterium*	*Bacillus*	*Micrococcus*	Enterobacteria†	Others‡
Hand line, Port Orchard	27	63·0	3·7	18·5	3·7	0·0	0·0	11·1	0·0	0·0
Otter trawl, Port Orchard	135	43·8	14·8	17·0	5·9	5·2	3·7	5·2	0·7	3·7
Inshore otter trawl, Golden Gardens	14	28·7	0·0	7·1	21·4	0·0	21·4	21·4	0·0	0·0
Beach seine, Golden Gardens	21	52·4	9·6	14·3	0·0	0·0	4·7	0·0	14·3	4·7
Purse seine, Gig Harbour	102	54·9	2·9	15·7	4·9	2·9	0·0	4·9	8·9	4·9

* Of all species combined. † *Aerobacter*, *Aeromonas*, *Proteus*.
‡ *Alcaligenes*, *Brevibacterium*, *Arthrobacter*, *Photobacterium*, yeasts.

It is generally accepted that the aerobic bacterial flora of marine fresh fish consists predominantly of *Pseudomonas* and *Achromobacter* spp. (*c.* 80%), with *Micrococcus* and *Flavobacterium* spp. making up the bulk of the remaining population, and it is clear from Table 3 that this was also true in the present work of fish caught by all methods except inshore otter trawling.

The flora of the line caught fish consisted entirely of representatives of *Pseudomonas*, *Achromobacter*, *Micrococcus* and *Flavobacterium*, and of gut group vibrios. Specimens obtained from deeper water by otter trawling yielded a flora which differed in that Gram-positive organisms such as *Corynebacterium* and *Bacillus* spp. and miscellaneous types were also present. The Gram-positive organisms can be considered to derive from the net and from the bottom mud (Spencer, 1959, 1960) and the higher proportion of gut group vibrios corroborates the finding of Shewan (1949) that trawled fish become contaminated by gut contents expressed among the fish during the hauling process. The inshore otter trawled samples, in which the number of fish per haul was much smaller, yielded greater numbers of *Bacillus*, *Micrococcus* and *Flavobacterium* only.

The largest numbers of enterobacterial types were encountered in the beach seine sample. These were mainly *Aerobacter*, *Proteus* and *Aeromonas* spp. and were most probably derived from both run-off and pollution occurring at Golden Gardens beach. However, the sample (21) is not sufficiently large for statistically significant conclusions to be drawn.

The purse seine samples did not differ greatly from the line caught samples and can be considered to yield a flora showing, in comparison with the other types of netting operations, the least amount of influence from handling. The incidence of *Corynebacterium* spp., enterobacteria and miscellaneous types on the purse seined fish would, no doubt, be related to the unavoidable handling during the netting and gaffing of the fish.

The factors involved in the quantitative and qualitative variations of the normal bacterial flora of marine fish have been discussed by Shewan (1944). For example, Lücke & Schwartz (1937) reported lower bacterial counts for the gut contents of herring than Shewan (1944) did for haddock and codling, and this is because the latter are bottom feeders whereas herring, as pelagic fish, feed on plankton. Lücke & Schwartz (1937) observed, furthermore, that the skin of whiting and cod caught singly by line yielded significantly lower bacterial counts than cod, saithe, red barsch, herring and mackerel caught by trawl net. But whereas the quantitative aspect is important, the qualitative composition of the flora is, perhaps, more significant in relation to spoilage. Excessive contamination of the freshly caught fish from gear,

Table 4. *The percentage incidence of certain biochemical and physiological characteristics among bacteria isolated from variously caught marine fish*

Character	Percentages of isolates with positive characters, from fish caught*				
	A	B	C	D	E
Motility	77·8	54·1	42·8	60·0	52·3
Growth at 0°	66·7	93·3	0·0	0·0	NT†
Growth at 37°	18·5	37·2	83·4	76·2	NT
Sensitivity to penicillin	25·9	24·8	70·0	25·0	25·5
Peptonization of litmus milk	33·3	20·9	NT	57·2	52·0
Liquefaction of gelatin	37·0	32·4	NT	89·5	65·0
Production of urease	37·0	27·4	NT	23·8	51·0
Reduction of nitrate	22·2	46·0	NT	28·6	32·4
Reduction of TMO‡	29·6	20·5	0·0	26·4	17·8
Hydrolysis of starch	7·4	19·8	14·3	10·0	10·8
Production of indole	14·8	5·3	NT	4·8	0·0
Voges-Proskauer reaction	22·2	13·4	NT	20·0	16·7
Methyl red test	14·8	44·7	NT	10·0	9·8
Production of H_2S	0·0	6·3	NT	23·8	19·4
Aerobic Hugh & Leifson test	74·1	73·6	NT	33·3	88·3
Anaerobic Hugh & Leifson test	7·7	26·4	NT	23·8	11·8
Acid from lactose	3·8	7·4	NT	23·8	19·6
Acid from maltose	7·4	20·5	NT	28·4	18·6
Acid from mannitol	0·0	10·9	NT	25·0	18·6
Acid from sucrose	3·7	10·9	22·2	20·0	30·4
Acid from galactose	3·7	13·6	33·3	31·6	26·4
Production of NH_3§	85·2	85·6	NT	NT	37·8
Catalase	86·4	94·6	91·6	95·3	NT
Kovacs oxidase	72·7	59·0	100	80·9	NT
Cytochrome oxidase	27·3	14·3	66·6	47·4	NT
Oxidation of gluconate	22·7	71·5	22·2	36·8	NT

* A, by hand line, Port Orchard; B, by otter trawl, Port Orchard; C, by inshore otter trawl, Golden Gardens; D, by beach seine, Golden Gardens; E, by purse seine, Gig Harbour. † N.T., not tested. ‡ TMO, trimethylamine oxide. § In peptone water.

deck or muds can be expected to alter the normal flora adversely, with reference to the keeping quality of the product. The bacterial populations deriving from contamination during the catching operation might cause more rapid deterioration. Since the several operations carried out in this study were on a pilot scale, with unusually careful handling of the fish, one would expect the magnitude of the effects of catching method to be several times greater in commercial operations.

The biochemical and physiological characteristics of the bacterial cultures are presented, in summarized form, in Table 4. The line caught and the purse seined fish samples probably reflect most clearly the bacterial flora of fish in their natural habitat. Variations imposed by the different locations of Port Orchard and Gig Harbour within Puget Sound might be expected, but as can be seen from Tables 3 and 4 only small differences were observed and these can be accounted for in terms of the handling fish receive in purse seining operations. The bacteria from these samples were predominantly milk peptonizing, gelatin liquefying, urea attacking and oxidase positive and did not ferment, but oxidized carbohydrates.

Pseudomonas spp. have been considered the most important spoilage types (Liston & Shewan, 1958; Castell & Anderson, 1948; Castell, Greenough & Jenkin, 1957). However, not all *Pseudomonas* spp. are of equal importance in this regard and there is some evidence that many of the species which occur on the freshly caught fish are of no significance in the actual spoilage process (Report, 1957). In view of this, it is difficult to evaluate the significance of the differences in the fish samples so far as spoilage is concerned, although the possible public health significance of the appreciable enterobacterial contamination of inshore fish is obvious. The wide range of biochemical properties of the bacteria from all fish samples indicates the diversity of possible pathways of degradation during spoilage.

The bacteria derived from otter trawl samples showed a higher incidence of fermentative types than those from the hand lined samples. In addition, it is interesting to note that this was not related to the occurrence of enterobacteria, but was possibly due to contamination of the fish by material from the sea bed stirred up by the catching operation. Disturbances of this type should be greatest in the case of otter trawling. However, according to the criteria of milk peptonization and gelatin liquefaction, the total proteolytic capacities of the two samples were approximately the same. The high incidence of proteolytic bacteria in the purse seined samples corresponded with a high occurrence of fluorescent pseudomonads, a type of bacterium not commonly associated with bottom muds.

From these observations, it does not seem that bottom mud contamination contributes significantly to the proteolytic capability of the bacterial flora. If proteolysis of this type is an important factor in fish spoilage, the effects of trawling on the bacterial flora may be of less significance in regard to spoilage than has been assumed in the past. However, the physical effects of trawling on the fish cannot be discounted. In view of the relatively incomplete understanding of the spoilage process and the bacteria involved in it, it would be unwise to disregard even minor variations in the flora which can be ascribed to catching methods. Such changes occur at the point of capture and influence the sequence of changes which take place during subsequent process operations.

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REFERENCES

ASCHEHOUG, V. & VESTERHUS, R. (1943). Investigations of the bacterial flora of fresh herring. *Zbl. Bakt.* (Abt. 2) **106**, 5.

BEDFORD, R. H. (1937). Bacteria in the gut of line caught halibut. *Progr. Rep. biol. Stas Nanaimo & Prince Rupert* no. 33, p. 23.

BLAKE, I. (1935). Some observations on the bacterial flora of the alimentary tract of certain Salmonidae. *Final Rep. Furuncul. Comm. Scot.* Edinburgh: H.M.S.O.

CASTELL, C. H. & ANDERSON, G. W. (1948). Bacteria associated with spoilage of cod fillets. *J. Fish. Res. Bd Can.* **7**, 370.

CASTELL, C. H., GREENOUGH, M. F. & JENKIN, N. L. (1957). The action of *Pseudomonas* on fish muscle: 2. Musty and potato-like odours. *J. Fish. Res. Bd Can.* **14**, 775.

CLEMENS, W. A. & WILBY, G. V. (1946). Fishes of the Pacific Coast of Canada. *Bull. Fish. Res. Bd Can.* no. 68.

COLWELL, R. R. (1961). Commensal bacteria of marine animals. A study of their distribution, physiology and taxonomy. Thesis, University of Washington, Seattle.

COLWELL, R. R. & LISTON, J. (1961). Taxonomic relationships among the pseudomonads. *J. Bact.* **82**, 1.

DELACY, A. C. & ENGLISH, T. S. (1954). Variations in beach seine samples caused by net length and repeated hauls. *Ecology* **35**, 18.

DYER, F. E. (1947). The microorganisms from Atlantic cod. *J. Fish. Res. Bd Can.* **7**, 128.

GABY, W. L. & FREE, E. (1958). Differential diagnosis of *Pseudomonas*-like microorganisms in the clinical laboratory. *J. Bact.* **76**, 442.

GEE, A. H. (1927). Bacteria concerned in the spoilage of haddock: preliminary report. *Contr. Canad. Biol.* **3**, 349.

GEE, A. H. (1930). Bacteria concerned in the spoilage of haddock. III. Further observations on the flora of live fish. *Contr. Canad. Biol.* **5**, 433.

GEORGALA, D. L. (1958). The bacterial flora of the skin of North Sea Cod. *J. gen. Microbiol.* **18**, 84.

GIBBONS, N. E. (1934*a*). The slime and intestinal flora of some marine fishes. *Contr. Canad. Biol.* **8**, 275.

GIBBONS, N. E. (1934*b*). Lactose-fermenting bacteria from the intestinal contents of marine fish. *Contr. Canad. Biol.* **8**, 291.

GRIFFITHS, F. P. (1937). A review of the bacteriology of fresh marine fishery products. *Food Res.* **2**, 121.

HARRISON, F. C. (1929). The discoloration of halibut. *Canad. J. Res.* **1**, 214.

HUGH, R. & LEIFSON, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J. Bact.* **66**, 24.

HUNTER, A. C. (1922*a*). A comparative study of spoilage in salmon. *Amer. Food J.* **17**, 19.

HUNTER, A. C. (1922*b*). The sources and characteristics of the bacteria in decomposing salmon. *J. Bact.* **7**, 85.

KISER, J. S. (1944). Effects of temperatures approximating 0°C upon growth and biochemical activities of bacteria isolated from mackerel. *Food Res.* **9**, 257.

KISER, J. S. & BECKWITH, T. D. (1942). Effect of fast freezing upon bacterial flora of mackerel. *Food Res.* **7**, 255.

KISER, J. S. & BECKWITH, T. D. (1944). A study of the bacterial flora of mackerel. *Food Res.* **9**, 250.

KOVACS, N. (1956). Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature, Lond.*, **178**, 703.

LISTON, J. (1954). A group of luminous and non-luminous bacteria from the intestine of flatfish. *J. gen. Microbiol.* **12**, i.

Liston, J. (1956). Quantitative variations in the bacterial flora of flatfish. *J. gen. Microbiol.* **15**, 305.

Liston, J. (1957). The occurrence and distribution of bacterial types on flatfish. *J. gen. Microbiol.* **16**, 205.

Liston, J. & Colwell, R. R. (1962). Host and habitat relationships of marine commensal bacteria. *Bact. Rev.* **26** (in press).

Liston, J. & Shewan, J. M. (1957). Bacteria brought into brines on fish. *Proc. 2nd Int. Food Symp. Microbiol., Cambridge*, p. 35.

Lücke, R. & Schwartz, W. (1937). Mikrobiologische Untersuchungen an Seefischen. *Arch. Mikrobiol.* **8**, 207.

Macleod, R. A., Onofrey, E. & Norris, M. E. (1954). Nutrition and metabolism of marine bacteria. I. Survey of nutritional requirements. *J. Bact.* **68**, 680.

Manual (1957). *Manual of Microbiological Methods*, of the Society of American Bacteriologists. New York: McGraw-Hill.

Margolis, L. (1953). The effect of fasting on the bacterial flora of the intestine of fish. *J. Fish. Res. Bd Can.* **10**, 62.

Nair, A. N. (1934). Spoilage of fresh fish with special reference to flatfishes. Thesis, University of Washington, Seattle.

Obst, M. M. (1919). A bacteriologic study of sardines. *J. infect. Dis.* **24**, 158.

Proctor, B. E. & Nickerson, J. T. R. (1935). An investigation of the sterility of fish tissues. *J. Bact.* **30**, 377.

Reay, G. A. & Shewan, J. M. (1949). The spoilage of fish and its preservation by chilling. *Advanc. Food Res.* **2**, 344.

Reed, G. B. & Spence, C. M. (1929). The intestinal and slime flora of the haddock: a preliminary note. *Contr. Canad. Biol.* **4**, 257.

Sanborn, J. R. (1930). Certain relationships of marine bacteria to the decomposition of fish. *J. Bact.* **19**, 375.

Sanborn, J. R. (1932). Marine bacteria commonly found in fresh fish. *J. Bact.* **23**, 349.

Shewan, J. M. (1938). The strict anaerobes in the slime and intestines of the haddock (*Gadus aeglefinus*). *J. Bact.* **35**, 397.

Shewan, J. M. (1944). The bacterial flora of some species of marine fish and its relation to spoilage. *Proc. Soc. agric. Bact.* p. 56.

Shewan, J. M. (1949). Some bacteriological aspects of handling, processing and distribution of fish. *J.R. sanit. Inst.* **69**, 394.

Shewan, J. M. (1962). The microbiology of sea-water fish. In *Fish as Food*, ed. Georg Borgstrom, New York: Academic Press, p. 487.

Shewan, J. M., Hodgkiss, W. & Liston, J. (1954). A method for the rapid differentiation of certain non-pathogenic, asporogenous bacilli. *Nature, Lond.*, **173**, 208.

Snow, J. E. & Beard, P. J. (1939). Studies on the bacterial flora of North Pacific salmon. *Food Res.* **4**, 563.

Spencer, R. (1959). The sanitation of fish boxes. I. The quantitative and qualitative bacteriology of commercial wooden fish boxes. *J. appl. Bact.* **22**, 73.

Spencer, R. (1960). The sanitation of fish boxes. II. The efficiency of various sanitizers in the cleaning of commercial wooden fish boxes. *J. appl. Bact.* **23**, 10.

Spencer, R. (1961). The occurrence of luminous bacteria in the intestinal tract of marine fish. *J. appl. Bact.* **24**, x.

Stewart, M. M. (1932). The bacterial flora of the slime and intestinal contents of the haddock (*Gadus aeglefinus*). *J. Mar. biol. Ass. U.K.* **18**, 35.

Tarr, H. L. A. (1954). Microbial deterioration of fish post mortem, its detection and control. *Bact. Rev.* **18**, 1.

Thjøtte, Th. & Sømme, O. M. (1943). The bacterial flora of normal fish. *Skr. norske VidenskAkad.* **4**, 1.

Venkataraman, R. & Sreenivasan, A. (1952). A preliminary investigation of the bacterial flora of the mackerels of the West Coast. *Indian J. Med. Res.* **40**, 529.

Venkataraman, R. & Sreenivasan, A. (1954). Bacteriology of inshore sea-water and of mackerels off Tellicherry (Malabar). *Proc. nat. Inst. Sci. India* **20**, 651.

Wilson, G. S. & Miles, A. A. (1955). Topley & Wilson's *Principles of Bacteriology and Immunity*, 4th ed. London: Edward Arnold.

Wood, E. J. F. (1940). Studies on the marketing of fresh fish in Eastern Australia. Part III. The bacteriology of spoilage of marine fish. *Pamphl. Coun. Sci. industr. Res. Aust.* no. 100, p. 1.

Wood, A. J. & Baird, E. A. (1943). Reduction of trimethylamine oxide by bacteria. *J. Fish. Res. Bd Can.* **6**, 194.
ZoBell, C. E. (1946). *Marine Microbiology.* Waltham, Mass.: Chronica Botanica Co.

(*Received* 27 *November*, 1961)

MEDREK, T. F. & BARNES, E. M. (1962). *J. appl. Bact.* **25** (2), 159–168.

THE DISTRIBUTION OF GROUP D STREPTOCOCCI IN CATTLE AND SHEEP

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SUMMARY: Numbers and types of Lancefield group D streptococci have been determined in samples from the colons of 17 cattle and 9 sheep. Mean total streptococcal counts of 8×10^4/g in cattle and 2×10^6/g in sheep were obtained. *Streptococcus bovis* was found in every sample and was the predominant species in 15 of the cattle and 6 of the sheep. Other group D streptococci (*Strep. faecalis*, *Strep. faecium* and *Strep. durans*) were rare in cattle, but in sheep they formed a significant proportion of the population. Of 60 *Strep. faecium*, *Strep. durans* and related strains, 51 fermented raffinose. Many of the strains of *Strep. faecium* were also atypical in that they fermented sorbitol and appreciably reduced tetrazolium in broth at pH 6·0.

Strep. bovis remained the predominant streptococcus in faeces samples from 4 dairy cows when they were tested again after an interval of 17 and 18 months.

WITH THE IMPROVED methods now available for the isolation and identification of faecal streptococci, interest has once more been focussed upon the distribution of these organisms in various animals. In early studies on ruminants, Winslow & Palmer (1910) observed that raffinose fermenting strains of streptococci appeared to be more abundant in bovine than in human faeces and that mannitol fermenting strains, which constituted a quarter of human faecal streptococci, were very rare in the faeces of cattle. Orla-Jensen (1919) described the species *Streptococcus bovis* and considered it to be the most frequently occurring faecal streptococcus in cattle, an opinion later supported by Ayres & Mudge (1923) and by Higginbottom & Wheater (1954). On the other hand Cooper & Ramadan (1955), Buttiaux (1958) and Wilssens & Buttiaux (1958) found this species only irregularly in cattle faeces and very seldom in sheep. Kjellander (1960) developed a selective medium for counting faecal streptococci which also differentiated the sorbitol fermenters, the inference being that these latter were mainly *Strep. faecalis* and its varieties *liquefaciens* and *zymogenes*. He examined faecal samples from 14 cattle, 10 calves and 10 sheep and found that the average (geometric mean) total streptococcal counts were 74,000/g for cows, 120,000/g for calves and 230,000/g for sheep, whereas the counts of sorbitol fermenting (*faecalis*) strains were 420/g, 8,000/g, and 3,400/g, respectively. Wilssens & de Vleeschauwer (1959) found a higher *Strep. faecalis* count in young calves than in adult cattle and suggested that this was due to the milk diet of the young calf. When the milk diet ceased, *Strep. faecalis* tended to disappear. Smith (1961) also found that young animals tend to have a higher proportion of *Strep. faecalis* strains in their faeces than do adult animals.

In the experiments described in this paper, the relative proportions of the different faecal streptococci have been determined in cattle and sheep, and the strains isolated have been examined to compare their properties with those of strains from other animals. The effects of time and the change from winter to summer feeding have also been determined in a single herd of cattle.

MATERIALS AND METHODS

Origin of samples

At a local abattoir samples were withdrawn aseptically from the colons of freshly slaughtered cattle and sheep at intervals over a period of one year. Other samples of freshly voided faeces were taken as aseptically as possible from 4 dairy cows of the herd belonging to the Cambridge University School of Veterinary Medicine.

Bacteriological examination

Enumeration of organisms

Preparation of dilutions. A sample of 1 or 2 g of faecal material was weighed into a wide necked 1 oz bottle containing 15 glass beads and emulsified with 9 or 18 ml of quarter-strength Ringer's solution. Decimal dilutions were then prepared using the same diluent and 0·025 ml of each of four consecutive dilutions was spread in duplicate over one quarter of Petri dishes containing the required agar medium.

Coli-aerogenes bacteria. These were counted on MacConkey's agar (Oxoid no. 3) which was incubated at 37° for 1 day.

Faecal streptococci (*Lancefield group D*). The thallous acetate-tetrazolium agar medium of Barnes (1956*a*) was used. It is important that the pH of the basal medium should not be below 6·0, as at pH 5·8 no growth occurs with certain strains of *Strep. bovis* and other faecal streptococci.

The plates were incubated aerobically for 24 hr at 37°. Colonies of *Strep. faecalis* and its varieties are generally medium sized (1–1·5 mm diameter) with red centres and white peripheries, but may also be dark red without the white periphery. *Strep. faecium*, *Strep. durans* and certain strains related to these two species form small to medium sized colonies varying from white through pink centred to uniformly pink. *Strep. bovis* strains characteristically form minute dark red colonies, but some strains classified as *Strep. bovis* form medium sized white colonies (Medrek & Barnes, 1958).

Selection and purification of isolates

The choice of strains for detailed study was based upon the appearance and size of colonies. Representatives of each colony type were taken from the highest dilution in which it appeared.

For the purification of isolates transfers were made into 0·1% thallous acetate broth (Barnes, 1956*a*) incubated for 24 hr at 37°, then on to tetrazolium-glucose agar (without thallous acetate) and incubated for 24 hr. The colonies were examined microscopically and were also tested for catalase to eliminate micrococci; but these were seldom found. All colonies, except those which were probably *Strep. bovis*, were subcultured in Hartley's digest broth; for colonies resembling those of *Strep. bovis* brain-heart infusion broth (Difco) was used.

Identification of species

Except for the following additions and modifications the methods used were those described by Barnes, Ingram & Ingram (1956).

Haemolysis. Hartley's digest agar was overpoured with a layer of digest agar containing 5% of horse blood. The plates were streaked with the organisms and examined after incubation at 37° for 24 and 48 hr. Final observations were made after overnight storage at 1°.

Aesculin hydrolysis. Medium B of Barnes *et al.* (1956) containing 0·1% of aesculin and 0·01% of ferric citrate was used. The aesculin and ferric citrate solutions were sterilized separately at 5 lb/in^2 for 30 min. The plates were incubated for 5 days and a positive reaction was indicated by blackening of the medium.

Slime production. Sucrose-gelatin agar plates were prepared as described by Niven, Smiley & Sherman (1941). Two sets of plates were streaked and incubated for 18 hr at 37°, one in an atmosphere of H_2 plus 10% of CO_2 and the other in air.

Starch hydrolysis. Medium B without glucose but containing 0·2% of soluble starch was used. The starch solution was sterilized separately by autoclaving at 5 lb/in^2 for 30 min. The plates were incubated for 5 days and zones of hydrolysis detected by flooding the plates with a 1 in 5 dilution of Gram's iodine.

Carbohydrate fermentation. Peptone water containing 1% of carbohydrate and Andrade's indicator was used.

Tetrazolium reduction at pH 6·0. The method of Barnes (1956*b*) was used, but the percentage of reduction to red formazan was recorded as follows: +, <10; ++, 10–20; +++, 20–30; ++++, >30.

Gelatin liquefaction. Cultures were incubated at 20° and examined after 14 days. Those strains which failed to grow at 20° (i.e. *Strep. bovis*) were incubated for 4 days at 37° and then chilled to reveal liquefaction.

Growth at 45°. Cultures were examined daily for up to 3 days.

RESULTS

The numbers and presumptive types of faecal streptococci

In order to determine both the total counts of faecal streptococci in cattle and sheep and the relative proportions of the main species present, thallous acetate-tetrazolium agar (TlTG) was used. Although *Strep. bovis* does not grow as well on this as on some of the other selective media, the colonies being very small, it has the considerable advantage of permitting an initial differentiation between species on the basis of colony size and tetrazolium reduction (see above). Seventeen samples taken from the colons of freshly slaughtered cattle and 9 from sheep were examined. At the same time coli-aerogenes bacteria were counted on a proportion of the samples.

Cattle

A summary of the total numbers and probable distribution of species of faecal streptococci from the 17 colon samples from cattle is given in Table 1. The total streptococcal count varied between 4×10^3 and 3×10^6/g (geometric mean, 8×10^4/g).

Strep. bovis was found in all the samples and was the predominant faecal streptococcus in 15 of them. Except for a single isolate at low dilution, *Strep. faecalis* and its varieties were not found. *Strep. faecium* and related strains were found in 8 samples only and in low numbers.

Five of the samples were tested for coli-aerogenes organisms, the counts being between $<2\times10^3$ and 5×10^4/g (geometric mean, $<10^4$/g).

Sheep

The results obtained with the 9 samples from the colons of sheep are also given in Table 1. The total streptococcal count varied between $1{\cdot}5\times10^5$ and 2×10^7/g (geometric mean, 2×10^6/g). *Strep. bovis* was again found in all the samples, and was predominant in 6 of them. *Strep. faecalis* and/or its varieties were found in 2 samples, and in one of these it was the predominant faecal streptococcus. *Strep. faecium* and related organisms were found in all the samples examined, often in significant numbers (geometric mean, *c.* 10^5/g). Judging from the variety of colony types the streptococcal flora appeared to be very heterogeneous.

Table 1. *The occurrence and probable numbers of the different species of faecal streptococci in the colons of cattle and sheep, as determined by counts and colonial appearance on thallous acetate-tetrazolium agar*

Source and no. of samples	Colony type on TITG agar	No. of samples positive	Log$_{10}$ (count/g wet wt)		
			Minimum	Maximum	Geometric mean*
Cattle 17	Minute red (*Strep. bovis*)	17	3·2	6·5	4·8
	Small to medium, white to pink (*Strep. faecium* type)	8	<2·3	6·2	<2·9
	Medium, red centred (*Strep. faecalis* and variants)	1	<2·3	2·3	<2·3
	Total colonies		3·6	6·5	4·9
Sheep 9	Minute red (*Strep. bovis*)	9	5·2	6·9	5·9
	Small to medium, white to pink (*Strep. faecium* type)	9	2·4	7·3	5·1
	Medium, red centred (*Strep. faecalis* and variants)	2	<1·3	6·7	<2·0
	Total colonies		5·2	7·3	6·3

* Calculated on the basis of the total number of samples.

Coli-aerogenes bacteria were found in all the 9 samples, the counts varying between 1×10^5 and 3×10^7/g (geometric mean, 4×10^6/g).

Confirmatory identification of streptococcal isolates

Representative strains of the different types of faecal streptococci from all the cattle and sheep samples were identified as far as possible using both serological and biochemical tests. The results are given below.

Strep. bovis *and related strains*

All the strains selected which formed minute red colonies on TlTG agar were found to conform with the characteristics of *Strep. bovis* as described by Orla-Jensen (1919). Altogether 152 of these isolates from cattle and sheep were examined physiologically and 148 serologically; they formed a remarkably homogeneous collection (Medrek & Barnes, 1962). One strain which appeared on TlTG agar as a small white colony was also identified as *Strep. bovis* but differed in a number of characteristics.

Table 2. *The differentiation of* 60 *strains of* Strep. faecium *and* Strep. durans (*and unrelated strains*) *isolated from cattle and sheep into groups*

Character	Nos. of samples and strains* and reactions† of						
	Strep. faecium, group			Related, group		*Strep. durans*, group	
	MA‡ (2, 2)	MAR‡ (5, 16)	MSAR‡ (6, 16)	1 (2, 2)	2 (8, 19)	Atypical (2, 4)	Typical (1, 1)
Haemolysis of horse blood	g	g (14) γ (2)	g (13) γ (3)	g	g (13) β (6)	g	β
Growth at 45°	+	+ (14) − (2)	+	+	+	+	+
Growth at pH 9·6	+	+	+ (14) − (2)	+	+	−	+
Survival at 60° for 30 min	+	+ (13) − (3)	+ (14) − (2)	+	+	+	+
Sucrose	A	A	A (14) − (2)	A	A	−	−
Mannitol	A	A	A	−	−	−	−
Sorbitol	−	−	A	−	−	−	−
Arabinose	A	A	A	−	−	−	−
Raffinose	−	A	A (15) − (1)	−	A	−	−
Litmus milk	rAC	rA (9) RA (5) RAC (2)	RA (7) RAC (7) rA (1) A (1)	rA	rA (18) RA (1)	rA	rA
Tetrazolium reduction in broth at pH 6·0§	−	+++	++ (12) + (1) − (3)	−	− (18) + (1)	−	+
Colony size	Medium	Small-medium	Medium	Medium	Medium	Small	Small
Appearance on TlTG agar	White	Pink	White-pink	White	White	White	White

* These are given at the head of each column of reactions, in brackets, with the no. of samples first.

† Reactions: +, positive; −, negative. Blood agar: g, greening; γ, no haemolysis; β, beta haemolysis. Fermentations: A, acid; r, slight reduction; R, strong reduction; C, clot. The figures in brackets indicate the nos. of strains with the specified reaction.

‡ The letters indicate the carbohydrates fermented: M, mannitol; A, arabinose; R, raffinose; S, sorbitol.

§ Tetrazolium reduction: −, none; +, <10%; ++, 10–20%; +++, 20–30%.

Strep. faecium, Strep durans *and related strains*

Sixty strains, which by their colonial appearance on tetrazolium agar were probably *Strep. faecium*, *Strep. durans* or related strains, were examined using the methods given above. They all belonged serologically to Lancefield's group D, and all grew on 40% bile agar, hydrolyzed aesculin and fermented lactose, glucose and salicin. They failed to ferment or hydrolyze starch, to produce typical growth on potassium tellurite-blood agar, and to liquefy gelatin; all grew at 10° and 45° (except for 2 strains) and in the presence of 6·5% salt. They could be differentiated into 7 main groups as shown in Table 2, mainly by differences in fermentation pattern and blood agar reactions. Three of the groups were designated *Strep. faecium* MA, MAR and MSAR, the letters indicating the carbohydrates fermented. Out of a total of 34 strains only 2 were typical (*Strep. faecium* MA, being strains which fermented mannitol and arabinose but not sorbitol or raffinose). Of the other 32 strains 31 fermented raffinose and 16 (*Strep. faecium* MSAR) also fermented sorbitol. Furthermore 10–30% of the tetrazolium was reduced to formazan by the majority of these *Strep. faecium* strains when they were grown in tetrazolium broth. The 2 strains designated 'related group 1' were similar to the 'unclassified type 1' strains described by Barnes *et al.* (1956), which differ from *Strep. faecium* in their failure to ferment arabinose. The 19 strains designated 'related, group 2' were separated from group 1 on their ability to ferment raffinose. Only one strain of a typical β-haemolytic *Strep. durans* was isolated. Four strains did not produce β-haemolysis and were differentiated from 'unclassified type 1' strains only by their failure to ferment sucrose and to grow at pH 9·6.

Strep. faecalis *and varieties* liquefaciens *and* zymogenes

In all, 7 strains which resembled *Strep. faecalis* or its varieties were isolated, 1 from cattle and 6 from sheep. None of these was typical. The single isolate from cattle was identified as *Strep. faecalis* var. *liquefaciens* and was serologically type H69D5 (Lancefield, 1941). Of the 6 isolates from sheep, all of which were Lancefield's type D76, 2 were identified as *Strep. faecalis* var. *liquefaciens* and 4 as *Strep. faecalis* var. *zymogenes.*

Distribution of the different species in cattle and sheep

Tables 3 and 4 give the origin and numbers of all the species (including varieties and related strains) of group D streptococci which were isolated from faecal material obtained from the colons of cattle and sheep. *Strep. bovis* was the predominant faecal streptococcus in both the cattle and sheep samples, though the numbers in the bovine samples were only one-tenth of those in the sheep.

It will be seen that only 8 out of the 17 cattle samples contained strains which fulfilled the criteria of Sherman (1937) for the 'enterococci' (i.e. *Strep. faecalis*, *Strep. faecium*, *Strep. durans* and related strains), and as only 17 organisms in all were isolated and examined it is difficult to draw any conclusions about the distribution; but all three of the *Strep. faecium* groups described above were found as well as both groups of the related strains.

In contrast to the results with cattle, 'enterococci' were found in all the sheep colon samples, not only in significant numbers but also with usually more than one species in a given sample. *Strep. faecium* was present in all but one sample and related strains of group 2 were regularly found. The single typical isolate of *Strep. durans* came from a sample (S-5) which also yielded atypical strains of this species

Table 3. *The numbers and distribution of the different kinds of group D streptococci* isolated from the colons of cattle*

Sample no.	No. of *Strep. bovis* (log$_{10}$ count/g)	*Strep. faecium* and related strains					
		Total no. (log$_{10}$ count/g)	No.§ of strains of groups				
			Strep. faecium‡			Related	
			MA	MAR	MSAR	1	2
1	4·4	<2·3					
2	3·9	<2·3					
3	4·9	6·2		5			
4	4·0	<2·3					
5	4·5	<2·3					
6	4·7	3·1					2
7	3·7	2·3	1				
8	3·9	2·3				1	
9	3·2	3·4					2
10	4·1	<2·3					
11	6·5	4·4					2
12	4·7	<2·3					
13	5·8	<2·3					
14	4·8	<2·3					
15	4·3	2·9			1		
16	4·3	4·1			2		
17	5·2	<2·3					
	4·8†	<2·9†					
		Total strains	1	5	3	1	6

* Sample 8 alone yielded 1 strain of *Strep. faecalis* or a variety thereof. It was not typical and the logarithm of its colony count was 2·3. † Geometric mean. ‡ See Table 2.
§ For clarity of presentation zeros have been omitted.

Table 4. *The numbers and distribution of the different kinds of group D streptococci isolated from the colons of sheep*

Sample no.	No. of *Strep. bovis* (log$_{10}$ count/g)	*Strep. faecium* and related strains								*Strep. faecalis* and varieties	
		Total no. (log$_{10}$ count/g)	No.‡ of strains of groups							Total no. (log$_{10}$ count/g)	No. of strains
			Strep. faecium†			Related		*Strep. durans*			
			MA	MAR	MSAR	1	2	Atypical	Typical		
1	6·8	5·9			6					<1·3	
2	5·8	4·1			4					<1·3	
3	6·9	6·9		5	2		1			2·0	2
4	5·7	4·3			1		4			<1·3	
5	5·9	7·3					6	2	1	<1·3	
6	5·3	3·8		2			2	2		<1·3	
7	6·1	4·6	1	1			1			<1·3	
8	5·3	5·6		3						6·7	4
9	5·3	2·4				1				<1·3	
	5·9*	5·1*								<2·0*	
		Total strains	1	11	13	1	13	4	1		6

* Geometric mean. † See Table 2. ‡ For clarity of presentation zeros have been omitted.

and a number of 'related, group 2' strains. All the strains from this sample were typed serologically, using the methods and typing sera of Sharpe & Fewins (1960), and whereas the six 'related, group 2' strains proved to be serological type 39, the two atypical *Strep. durans* strains were serological type 37. When the β-haemolytic strain of *Strep. durans* was tested it was also serological type 37, but had lost its β-haemolytic property.

Effect of diet

Preliminary studies were made to see whether there was any change in the numbers and kinds of faecal streptococci in the faeces of four dairy cows in a single herd following the change from summer to winter diets. Unfortunately more than a year elapsed between the first and second of the two tests, whereas there was only a difference of one month between the winter diet and the transitional diet. The results on the four cows (Susan, Prune, Proxy and Nautch) are given in Table 5.

Table 5. *The influence of diet and passage of time on the numbers of three species of faecal streptococci in the faeces of cows*

Date sampled	Diet*	Group D species	No., as $\log_{10}$ (count/g wet wt of faeces), from animal no.‡			
			1	2	3	4
Oct. '57	a	*Strep. bovis*†	6·6	5·2	5·7	6·1
Mar. '59	b		7·0	6·1	6·2	6·7
Apr. '59	c		5·9	6·6	5·1	6·4
Oct. '57	a	*Strep. faecium* type†	2·0	<1·7	<1·7	<1·7
Mar. '59	b		<1·7	<1·7	<1·7	<1·7
Apr. '59	c		<1·7	<1·7	<1·7	<1·7
Oct. '57	a	*Strep. faecalis*†	<1·7	<1·7	<1·7	<1·7
Mar. '59	b		<1·7	<1·7	<1·7	<1·7
Apr. '59	c		<1·7	<1·7	<1·7	<1·7

* Diets: a, summer (grass and grain); b, winter (hay, silage and grain); c, transition between summer and winter.

† Determined from the appearance of colonies on thallous acetate-tetrazolium agar at pH 6·0.

‡ Identities of animals: 1, Susan; 2, Prune; 3, Proxy; 4, Nautch.

The samples were counted as previously, using thallous acetate-tetrazolium agar, and it was found that *Strep. bovis* continued to be the predominant faecal streptococcus; *Strep. faecalis* and its varieties were not found and *Strep. faecium* or related strains were found in one sample only, that from Susan on a winter diet.

DISCUSSION

By using a differential medium it has been possible to analyze the minor as well as the major components of the faecal streptococci flora in cattle and sheep. *Strep. bovis* has been confirmed as the dominant species in both animals; details of the strains isolated have been given by Medrek & Barnes (1962). When considering *Strep. faecium* and related strains, one of the most interesting observations was the large number (50 out of 60) of raffinose fermenting strains present. The observation of Winslow & Palmer (1910) of a high proportion of raffinose fermenting strains in the faeces of cattle could therefore apply to these organisms as well as to *Strep. bovis*. The *Strep. faecium* strains were also remarkable in that 16 out of 60 fermented sorbitol as well as raffinose. Similar strains were not found by Barnes & Ingram (1955) in pigs nor have any been found by them in human faeces (unpublished data), but among strains sent by Dr. H. Williams Smith three gave similar reactions and were obtained from the faeces of a dog and a rabbit and off the back of a piglet. Wilssens & de Vleeschauwer (1959) reported an aberrant sorbitol fermenting strain of *Strep. faecium* from their cattle but did not state whether it also fermented raffinose. A number of the *Strep. faecium* strains were much more reducing in tetrazolium broth than usual and in addition reduced litmus milk. Although some of the *Strep. faecium* strains isolated from pigs by Barnes & Ingram (1955) actively reduced litmus milk they did not reduce the tetrazolium broth at pH 6·0 when retested recently (unpublished data).

The difficulties of classifying *Strep. durans* and related strains are once more illustrated in this work, and the whole relationship of *Strep. durans* to *Strep. faecium* will be clarified only when more information is available on the serology and physiology of a large number of isolates from different sources.

The *Strep. faecalis* strains isolated were of interest in that they were of the same serological types (H69D5 and D76) as those which are so frequently found in man. The low numbers of *Strep. faecalis* strains found, particularly in cattle, confirm the findings of others that these organisms are rare once the animals are old enough to be on a normal diet of grass, hay or grain.

Very little difference was found in the samples from cattle whether the animals were on summer, winter, or transitional diets. It is probable that a very considerable dietary change is necessary before a change will occur in either the numbers or types of faecal streptococci present, as for example the change from the milk diet of young animals to adult food.

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REFERENCES

AYRES, S. H. & MUDGE, C. S. (1923). Streptococci of faeces and mouth of cows. V. Studies of the streptococci. *J. infect. Dis.* **33**, 155.

BARNES, E. M. (1956*a*). Methods for the isolation of faecal streptococci (Lancefield group D) from bacon factories. *J. appl. Bact.* **19**, 193.

BARNES, E. M. (1956*b*). Tetrazolium reduction as a means of differentiating *Streptococcus faecalis* from *Streptococcus faecium* (Orla-Jensen). *J. gen. Microbiol.* **14**, 57.

BARNES, E. M. & INGRAM, M. (1955). The identity and origin of faecal streptococci in canned hams. *Ann. Inst. Pasteur, Lille*, **7**, 115.

BARNES, E. M., INGRAM, M. & INGRAM, G. C. (1956). The distribution and significance of different species of faecal streptococci in bacon factories. *J. appl. Bact.* **19**, 204.

Buttiaux, R. (1958). Les streptocoques fécaux des intestins humains et animaux. *Ann. Inst. Pasteur* **94**, 778.

Cooper, K. E. & Ramadan, F. M. (1955). Studies in the differentiation between human and animal pollution by means of faecal streptococci. *J. gen. Microbiol.* **12**, 180.

Higginbottom, C. & Wheater, D. W. F. (1954). The incidence of *Streptococcus bovis* in cattle. *J. agric. Sci.* **44**, 434.

Kjellander, J. (1960). *Enteric Streptococci as Indicators of Fecal Contamination of Water.* Copenhagen: Ejnar Munksgaard.

Lancefield, R. C. (1941). Specific relationship of cell composition to biological activity of haemolytic streptococci. *Harvey Lect.* Series 36, 251.

Medrek, T. F. & Barnes, E. M. (1958). A note on the growth of *Streptococcus bovis* in thallous acetate and tetrazolium media. *J. appl. Bact.* **21**, 79.

Medrek, T. F. & Barnes, E. M. (1962). Biochemical and serological properties of strains of *Streptococcus bovis* and related organisms. *J. appl. Bact.* **25**, 169.

Niven, C. F. (Jr.), Smiley, K. L. & Sherman, J. M. (1941). The production of large amounts of a polysaccharide by *Streptococcus salivarius*. *J. Bact.* **41**, 479.

Orla-Jensen, S. (1919). *The Lactic Acid Bacteria.* Copenhagen: A. F. Host & Son.

Sharpe, M. E. & Fewins, B. G. (1960). Serological typing of strains of *Streptococcus faecium* and unclassified group D streptococci isolated from canned hams and pig intestines. *J. gen. Microbiol.* **23**, 621.

Sherman, J. M. (1937). The streptococci. *Bact. Rev.* **1**, 1.

Smith, H. Williams (1961). The development of the bacterial flora of the faeces of animals and man: the changes that occur during ageing. *J. appl. Bact.* **24**, 235.

Wilssens, A. & Buttiaux, R. (1958). Les bactéries de la flora fécale de la vache saine. *Ann. Inst. Pasteur* **94**, 332.

Wilssens, A. & de Vleeschauwer, A. (1959). Enterococci in calves' faeces. *J. appl. Bact.* **22**, vii.

Winslow, C. A. E. & Palmer, G. T. (1910). A comparative study of the intestinal streptococci from the horse, cow and man. *J. infect. Dis.* **7**, 1.

(*Received* 19 *January*, 1962)

MEDREK, T. F. & BARNES, E. M. (1962). *J. appl. Bact.* **25** (2), 169–179.

THE PHYSIOLOGICAL AND SEROLOGICAL PROPERTIES OF *STREPTOCOCCUS BOVIS* AND RELATED ORGANISMS ISOLATED FROM CATTLE AND SHEEP

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SUMMARY: The physiological and serological properties of 153 strains of *Streptococcus bovis* isolated from cattle and sheep have been studied. With 12 specific antisera it was possible to type 75 of the isolates. Neither the fermentation of arabinose nor of raffinose was related to type specificity. The only strain fermenting mannitol was serologically distinct from all the other strains, and the type antigen was probably located on the cell wall whereas with the other 11 types it was capsular.

The distribution of these types in cattle and sheep has been determined together with their changes in individual cows sampled on three occasions over 18 months.

IN RECENT STUDIES of the group D streptococci of the colon and faeces of cattle and the colon of sheep Medrek & Barnes (1962) confirmed the constant presence and general predominance of *Streptococcus bovis*. The species *Strep. bovis* was first described by Orla-Jensen in 1919, who also differentiated a closely related species *Strep. inulinaceus*. Following a number of investigations Sherman (1937) concluded that there was little justification for considering *Strep. inulinaceus* as a species distinct from *Strep. bovis* and in the 7th edition of Bergey's *Manual* (Breed, Murray & Smith, 1957) it is omitted.

Strep. bovis possesses the group D antigen in common with *Strep. faecalis*, *Strep. faecium* and *Strep. durans* (Sherman, 1938; Shattock, 1949) but it differs from these species in many characteristics: it does not grow at pH 9·6, in the presence of 6·5% salt or at 10°, and is more heat sensitive and fails to produce ammonia from arginine (Niven, Smiley & Sherman, 1942).

As more strains of *Strep. bovis* are isolated from sources other than the alimentary tract of ruminants, it becomes increasingly evident that the species as now constituted is not a well defined entity. Dain, Neal & Seeley (1956) studied 87 strains from various sources with particular attention to mannitol fermentation and haemolysis of blood and to slime production on sucrose agar in an atmosphere containing CO_2. They found that 71 strains consistently produced slime and never fermented mannitol, whereas the strains fermenting mannitol produced no slime. In a further study of starch hydrolyzing strains, Seeley & Dain (1960) concluded that one of the most important diagnostic characters for the species was the ability to produce slime (dextran). In the absence of this character it was very difficult to differentiate *Strep. bovis* from the lactose negative species *Strep. equinus*.

Previously most of the serological studies with *Strep. bovis* have been concerned with the group D antigen, although some serological types within the group have been described. MacPherson (1953), in studies of the amylolytic rumen streptococci which were later identified as *Strep. bovis*, prepared several type specific antisera and demonstrated a capsular swelling reaction with homologous sera for her capsulated strains. The capsular material was shown by Hobson & MacPherson (1954) to be a type-specific polysaccharide. Of 20 strains of human origin 14 were identified by Sharpe & Shattock (1952) as belonging to one or other of 4 serological types. The serological complexity of *Strep. bovis* was indicated by Perry & Briggs (1955) and Perry, Newland & Briggs (1958) who found common type antigens with groups E and N.

A number of the strains of *Strep. bovis* which were isolated by Medrek & Barnes (1962) have now been examined physiologically and serologically to obtain a clearer understanding of the ecological relationships of these organisms and to assess the taxonomic importance of the various fermentation reactions by relating these to serological types. The results are presented in this paper.

METHODS

Sources of streptococci

In addition to the strains isolated by the authors from cattle and sheep and described below, the following strains were also used: *Strep. bovis* strains 14M4, 22M2, 24M4 and I (Bailey & Oxford, 1958), kindly supplied by Dr. S. O. Mann; B195 by Dr. S. D. Elliott; Dorritt 9, Pearl 11 and Rosalie 20 (Shattock, 1949) by Dr. M. E. Sharpe; strains of known serological type belonging to various species within group D—D76, H69D5 and C1 (Lancefield, 1941), D10 and D15 (Elliott, 1957) supplied by Dr. S. D. Elliott; strains S182, B26, N42, S194, S112, N55, B74, S98, L6, H6, H24 (Sharpe & Shattock, 1952) kindly supplied by Dr. M. E. Sharpe; strains CH1, CH3, HGH511, GE4B, N/H2, N/H4, N/H53, N/H64, P3, P7, P/1/12, P/6/4, P/10/1, P/14/6, G3, P/14/10, P/16/5 and P/17/8 (Sharpe & Fewins, 1960); strains W4 and W6 (Perry & Briggs, 1955), NS5, NS7 and NS56 (Perry, Newland & Briggs, 1958) kindly supplied by Dr. C. A. E. Briggs.

Physiological tests

The methods used were those described by Barnes, Ingram & Ingram (1956) and Medrek & Barnes (1962).

Serological tests

A total of 149 strains isolated by Medrek & Barnes (1962) were examined serologically.

Selection of type strains. In all, 12 type sera were prepared from strains C3, C33, C36, C48, C101, C106, CY8, S2/5b, S5, S19, S63 and S105. These types have been numbered arbitrarily 1–12 but it is recognized that the numbers will be changed when international agreement is reached on the type numbering for group D. The selection of type strains was based mainly upon physiological differences which are described in detail below, but care was also taken to ensure that they represented both cattle and sheep isolates.

Preparation of type sera. Organisms grown for 18 hr in 250 ml of brain heart infusion broth (Difco) were washed twice in 0·85% (w/v) saline and suspended in

12·5 ml of 2·0% (w/v) formaldehyde and stored at 4° for 3 to 4 days. They were then centrifuged, washed in saline and resuspended in 5 ml of saline. These vaccines were stored at 4° until required for inoculation into rabbits.

All inocula were made up to a final volume of 0·5 ml with saline. The first inoculation consisted of 0·1 ml of vaccine as a sensitizing dose. After the lapse of a week, inoculations were then made on three successive days in each of three successive weeks with 0·1 ml of vaccine used each time the first week, 0·2 ml the second week and 0·25 ml the third week. It was subsequently found that for the routine preparation of *Strep. bovis* type sera two series each consisting of 3 inoculations (after the sensitizing dose) were sufficient to produce potent antisera. The total number of streptococci injected into individual rabbits varied from $2{\cdot}4 \times 10^{10}$ to 10^{11}. Merthiolate (1:10,000) was added as a preservative. Bulk quantities of sera were stored at −20°, small aliquots for current use were stored at 1°.

Precipitin tests. Hydrochloric acid extracts were prepared by the method of Lancefield (1933). The organisms were generally grown in 10 ml of Todd-Hewitt broth, but occasionally 1% glucose-Lemco broth was used. Incubation was for 24 hr at 37°. The cells were centrifuged, resuspended in 0·5 ml of N/15 HCl in 0·85% NaCl and adjusted to pH 2–3. The suspensions were then heated in a boiling water bath for 10 min, shaken at intervals of 3 min and cooled and neutralized without delay.

The capillary technique of Swift, Wilson & Lancefield (1943) was used for the precipitin tests with tubes having an internal diameter of 1·0 mm. The sera were of such potency that positive reactions could be read within 1 min at room temperature.

RESULTS

Physiological and serological properties of Strep. bovis *isolates*

Physiological characteristics of Strep. bovis *isolates.* There was considerable physiological uniformity among the 153 strains of *Strep. bovis* which were isolated by Medrek & Barnes (1962) from 15 colon and 12 faecal samples from cattle and from 9 colon samples from sheep. All the strains tested were shown to possess the group D antigen, they all grew on 40% bile-blood agar but did not grow on 1 in 2,500 potassium tellurite agar. They all grew at 45°, but not at 10°, neither did they grow in the presence of 6·5% salt, or at pH 9·6; no strain consistently survived 60° for 30 min but the results were variable. None was positive for tyrosine decarboxylase or arginine hydrolysis. All the strains hydrolyzed aesculin and fermented lactose, sucrose and inulin and hydrolyzed and fermented starch, but none fermented glycerol or sorbitol. The strains could be separated by the reactions given in Table 1. A total of 152 strains produced greening on horse blood agar and grew as minute red colonies on tetrazolium agar at pH 6·0. They produced only slight acidity or little change in litmus milk, and failed to ferment mannitol. They possessed large capsules and generally produced copious quantities of slime on sucrose-gelatin agar plates incubated in an atmosphere of H_2 containing 10% of CO_2. Of these strains 100 (designated ARS) fermented arabinose, starch and raffinose; and 51 (RS) failed to ferment arabinose. One strain (S) fermented starch but failed to ferment either arabinose or raffinose. One strain (MRS) differed considerably from the others in that it was not capsulated, had no action on horse blood agar, grew as small white colonies on tetrazolium agar, formed acid and clot in litmus milk, and fermented mannitol, raffinose and starch but not arabinose.

Table 1. *The differences in physiological characters of 153 isolates of* Strep. bovis

Physiological group	No. of strains	Test*					Colony size and colour†
		Haemolysis	Mannitol	Arabinose	Raffinose	Litmus milk	
RS	51	g	—	—	A	a or NC	Minute, red
ARS	100	g	—	A	A	a or NC	
S	1	g	—	—	—	a	Minute, red
MRS	1	γ	A	—	A	AC	Small, white

* g, greening of blood; γ, no haemolysis; A, acid; a, slight acid; C, clot; NC, no change.
† On thallous acetate-tetrazolium agar.

Serological type strains. The two single isolates, MRS and S (Table 1), together with 7 ARS strains and 3 RS strains, were used for the preparation of antisera. In addition to the properties given above the strains all fermented maltose, mannose, dextrin and glycogen but did not ferment adonitol, dulcitol, inositol, rhamnose or xylose. No β-haemolysis occurred on horse blood agar plates incubated in an atmosphere of CO_2 (Dain, Seeley & Neal, 1957). Other tests which differentiated these organisms are given in Table 2.

Specificity of type sera. With one exception, tests with heterologous extracts showed the absence of cross reactions; this exception was with the extract of type 6 which showed a nonreciprocal cross reaction with the type 5 serum. Repeated absorptions of the type 5 serum with type 6 organisms significantly reduced, but did not completely remove, the cross reaction. This difficulty in absorption was in direct contrast to the extreme facility (only one absorption required) with which the sera of types 5 and 6 were absorbed with their homologous type strains.

Relation of Strep. bovis *type strains to other type strains of group D streptococci.* Nine strains of *Strep. bovis* (W4, W6, NS5, NS7, NS26, S182, S194, S112 and H6) which had been used by other workers for the preparation of type sera were examined to determine the presence of any possible common serological types. None of the 12 type sera prepared above reacted with these strains.

The type sera were also tested against the 30 other type strains of group D streptococci listed above (see Methods). None reacted with the *Strep. bovis* type sera.

Location of the type antigen. The twelve type strains of *Strep. bovis* were first examined by the technique of Duguid (1951) for the presence of capsules. Very large capsules were observed with all the type strains except the mannitol fermenting strain, type 5. The organisms were then tested with the type sera for the specific capsular reaction and this was found to be positive with all except type 5. It is thus probable that eleven of the type sera prepared in this study were prepared for the capsular antigens.

Following the method described by Elliott (1960) the type 5 strain was examined for the location of the type antigen. It was found that after disruption of the cells and the separation and repeated washing of the cell walls, these still gave a strong type reaction when extracted with lysozyme, thus suggesting that the antigen was associated with the cell wall.

Table 2. *Some differences in the physiological characters* of the* Strep. bovis *type strains*

Serological type	Strain No.†	Horse blood	Mannitol	Arabinose	Raffinose	Trehalose	Litmus milk	Slime production‡		Colony size and colour
								Without CO$_2$	With CO$_2$	
1	C3	g	—	—	A	A	a	−	+	Minute red
2	C33	g	—	A	A	—	a	+	+++	Minute red
3	C36	g	—	A	A	—	a	−	++	Minute red
4	C48	g	—	—	A	—	a	−	++	Minute red
5	C101	γ	A	—	A	A	AC	−	−	Small white
6	C106	g	—	—	—	—	a	−	−	Minute red
7	CY8	g	—	A	A	—	a	−	+++	Minute red
8	S2/5b	g	—	—	A	—	NC	−	++	Minute red
9	S5	g	—	A	A	—	NC	−	+++	Minute red
10	S19	g	—	A	A	—	NC	−	++	Minute red
11	S63	g	—	A	A	A	a	−	++	Minute red
12	S105	g	—	A	A	—	NC	±	+++	Minute red

* Characters: g, greening; γ, no haemolysis; A, acid; a, slight acid; C, clot; NC, no change.

† Strains designated C were from cattle and those designated S from sheep.

‡ Slime production: —, negative; ±, trace; +, discrete slimy colonies; ++, very slimy colonies; +++, copious quantities (no discrete colonies).

Relationship betwen serological type and physiological characteristics

All the strains of *Strep. bovis* tested with the 12 type sera are given in Table 3 which shows their ability to ferment arabinose, raffinose, starch and mannitol in relation to the type strains. Out of a total of 149 strains only 75 could be typed. The type 5 strain which fermented mannitol was serologically distinct from the other 148 strains which failed to ferment mannitol. Except in this instance no complete correlation was found between physiological and serological characteristics. For example, 3 raffinose fermenting strains (originating from 3 different animals) reacted with the type 6 antiserum prepared from the strain which failed to ferment raffinose. Four strains which did not ferment arabinose were of the same serological types (types 3, 7 and 11) as strains which did ferment arabinose. One strain which fermented arabinose was of the same serological type (type 4) as strains which did not.

Table 3. *The relationship between serological type and the ability to ferment mannitol, arabinose, raffinose and starch*

Strains*	Number of strains of serological type												Untyped strains	Total
	1 (RS)*	2 (ARS)	3 (ARS)	4 (RS)	5 (MRS)	6 (S)	7 (ARS)	8 (RS)	9 (ARS)	10 (ARS)	11 (ARS)	12 (ARS)		
RS	2	0	1	14	0	3	1	4	0	0	2	0	19	46
ARS	0	2	3	1	0	0	26	0	7	2	2	3	55	101
S	0	0	0	0	0	1	0	0	0	0	0	0	0	1
MRS	0	0	0	0	1	0	0	0	0	0	0	0	0	1
Total	2	2	4	15	1	4	27	4	7	2	4	3	74	149

* RS, fermentation of raffinose and starch; ARS, fermentation of arabinose, raffinose and starch; S, fermentation of starch; MRS, fermentation of mannitol, raffinose and starch.

Distribution of types in cattle and sheep

The distribution of the typed strains of *Strep. bovis* isolated from the colon of cattle and sheep is shown in Tables 4 and 5. Of the 29 cattle strains, 14 were typed and these originated from 7 animals. Type 4 was found in four animals and type 6 was found in three. In at least 6 cases where two or more strains were isolated there must have been at least two serological types and in one animal (no. 1) there were at least 4 types among the 5 strains isolated.

Table 4. *The distribution of serologically typed strains of* Strep. bovis *isolated from the colons of cattle*

Animal no.	$\log_{10}$ *Strep. bovis*/g	No.* of strains of serological types							Total
		3	4	5	6	8	11	Untyped†	
1	4·4		1			1	2	1	5
2	3·9				1			1	2
3	4·9		2‡						2
4	4·0		2		1				3
5	4·5							1	1
6	4·7		1					1	2
7	3·7							1	1
8	3·9							1	1
10	4·1							1	1
11	6·5							2	2
13	5·8							1	1
14	4·8							2	2
15	4·3			1‡				1	2
16	4·3							2	2
17	5·2	1			1‡				2
Total	4·6§	1	6	1	3	1	2	15	29

* For clarity of presentation zeroes have been omitted.

† No reaction with type sera 1–12. ‡ Origin of type strain. § Geometric mean.

Table 5. *The distribution of serologically typed strains of* Strep. bovis *isolated from the colons of sheep*

Animal no.	Log_{10} *Strep. bovis*/g	No.* of strains of serological types						Total
		8	9	10	11	12	Untyped†	
1	6·8		7‡					7
2	5·8			2‡			5	7
3	6·9	1‡					4	5
4	5·7					1	4	5
5	5·9				2‡		1	3
6	5·3						5	5
7	6·1					1	5	6
8	5·3						2	2
9	5·2					1‡	6	7
Total	5·9§	1	7	2	2	3	32	47

* For clarity of presentation zeroes have been omitted.

† No reaction with type sera 1–12. ‡ Origin of type strain. § Geometric mean.

Only 15 out of 47 isolates from sheep were typed with 5 out of the 12 type sera. The typed strains were isolated from 7 out of the 9 animals, but only type 12 was found in more than one animal. Types 8 and 11 were common both to cattle and sheep.

Distribution in a single herd

Preliminary studies have been made on the effect of diet and/or passage of time on the constancy of serological types in individual animals. The results obtained with 4 cows which were first tested in the autumn of 1957 and then re-examined 17 and 18 months later are given in Table 6. In only one instance (Prune) was the same serological type found after a lapse of 17 months. However, at least one common type was isolated from all the animals after a lapse of 1 month. Type 7 was predominant, being found in all the animals at least once and in three of them on two occasions. Several serological types again occurred together in a single faecal

Table 6. *The distribution of serological types of* Strep. bovis *in the faeces of 4 cows in a single herd, in relation to diet and passage of time*

Animal sampled	Date sampled	Diets**	Log_{10} *Strep. bovis*/g	No.* of strains of serological types								Total
				1	2	3	4	6	7	8	Untyped†	
Susan	Oct. '57	a	6·6	1							1	2
	Mar. '59	b	7·0						9			9
	Apr. '59	c	5·9				3		1		4	8
Prune	Oct. '57	a	5·2		2‡	1‡						3
	Mar. '59	b	6·1			1			5	1	1	8
	Apr. '59	c	6·6			1			1		6	8
Proxy	Oct. '57	a	5·7	1‡							1	2
	Mar. '59	b	6·2						7‡	1		8
	Apr. '59	c	5·1						3		4	7
Nautch	Oct. '57	a	6·1								1	1
	Mar. '59	b	6·2				3	1			5	9
	Apr. '59	c	6·4				3		1		4	8
	Total		6·1§	2	2	3	9	1	27	2	27	73

* For clarity of presentation zeroes have been omitted.

† No reaction with type sera 1–12. ‡ Origin of type strain. § Geometric mean.

** Diets: a, summer (grass and grain); b, winter (hay, silage and grain); c, transition between summer and winter.

sample. Although four of the 12 type sera were prepared with strains isolated from this herd, it was still only possible to type 46 out of the 73 strains isolated; this suggests that a large number of types must exist.

A study of strains of Strep. bovis *from other sources*

The single mannitol fermenting strain was so different from the other 152 strains, both serologically and physiologically, that it was of interest to compare it with strains isolated by other workers. Sixteen strains (Table 7) some of which fermented mannitol, were examined. All the strains belonged serologically to group D, grew on 40% bile and did not grow on 1 in 2,500 potassium tellurite-blood agar; they did not grow at 10°, nor in 6·5% salt; they did not survive heating at 60° for 30 min, but grew at 45°; they hydrolyzed aesculin, fermented lactose, raffinose and salicin but did not ferment glycerol and sorbitol; they did not hydrolyze arginine or decarboxylate tyrosine. The differences are shown in Table 7. The strains Dorritt 9, Pearl 11, Rosalie 20, NS7 and NS26, isolated from bovine faeces and the rumen, closely resembled the 100 strains (ARS) shown in Table 1; two of these strains (Dorritt 9 and Rosalie 20) were found to be of type 11, the homologous strain of which was isolated from the sheep colon. Strains I, W4, W6 and NS5 from the bovine rumen resembled the 51 strains (RS) isolated.

Table 7. *Some differences in physiological characters* of various strains of* Strep. bovis

Strain	Source	Horse blood	Fermentation of Manitol	Arabinose	Inulin	Starch	Starch hydrolysis	Litmus milk (24 hr)	Colony size and colour‡
Dorritt 9	Bovine faeces	g	—	A	A	A	+	A	Minute red
Pearl 11		g	—	A	A	A	+	A	
Rosalie 20		g	—	A	A	A	+	A	
NS3	Bovine rumen	g	—	A	A	A	+	A	
NS26		g	—	A	A	A	+	A	
I		g	—	—	A	A	+	A	
W4		g	—	—	A	A	+	A	
W6		g	—	—	A	A	+	A	
NS5		g	—	—	A	A	+	A	
14M4	Sheep's rumen	γ	A	—	A	A	+	AC	Small, slightly pink or white
22M2		γ	A	—	A	A	+	AC	
22N4		γ	A	—	A	A	+	AC	
B195	Human endocarditis	γ	A	—	A	A	+	AC	
S112	Human faeces	g	—	—	—	—	—	AC	Small, slightly pink
S194		g	—	—	—	—	—	AC	
S182		g	—	—	—	—	—	AC	

* Characters: g, greening; γ, no haemolysis; A, acid; C, clot; —, negative; +, hydrolysis in 5 days.

‡ On thallous acetate tetrazolium agar.

Strains 14M4, 22M2 and 24M4 isolated from the rumen and strain B195 from human endocarditis all resembled the single mannitol fermenting strain isolated (Table 1), but were serologically distinct. A further group of 3 strains (S112, S194 and S182 isolated from human faeces by Sharpe & Shattock, 1952) differed from the other *Strep. bovis* strains particularly in their failure to ferment mannitol, arabinose, starch and inulin. Shattock (pers. comm.) states that when first isolated these strains gave some hydrolysis of starch.

DISCUSSION

All of the 153 strains of *Strep. bovis* examined were clearly differentiated from the other species within group D (*Strep. faecalis*, *Strep. faecium* and *Strep. durans*) as they fermented both starch and inulin, failed to grow at pH 9·6, in 6·5% salt or at 10° and did not hydrolyze arginine. One hundred and fifty-two strains closely resembled each other in their physiological and serological properties and were apparently similar to the slime producing starch hydrolyzing strains of Seeley & Dain (1960). They differed from the original description given for *Strep. bovis* by Orla-Jensen (1919) in that casein was not attacked and the fermentation of arabinose was variable.

The single mannitol fermenting strain (C101, type 5) differed in a number of ways. It was not capsulated and did not produce dextran when grown on sucrose-gelatin agar in an atmosphere of H_2 containing 10% CO_2. It also produced acid and clot in litmus milk, grew more readily on ordinary nutrient media and was less susceptible to acid conditions than the other strains. It resembled the mannitol fermenting strains (14M4, 22M2 and 24M4) received from Dr. Mann (Table 7) although it was of a different serological type. Mann, Masson & Oxford (1954), Dain, Neal & Seeley (1956) and Seeley & Dain (1960) have drawn attention to the differences between the mannitol fermenting strains and the typically capsulated slime producing ones and it is evident that there are at least two main divisions within the species *Strep. bovis* as now characterized.

The preparation of typing sera enabled a further assessment to be made of the relevance of the various fermentation tests. The single mannitol fermenting strain (type 5) was serologically distinct from all other strains, with the type antigen probably associated with the cell wall as was found by Elliott (1960) for some *Strep. faecalis* types. In contrast, the other strains of *Strep. bovis* possessed large capsules which appeared to be serologically type specific, but it was evident that failure to ferment raffinose or arabinose was not a diagnostic character when used alone.

The immunological data clearly indicate a multiplicity of serological types in *Strep. bovis*. This general conclusion was confirmed by the fact that all 12 types were distinct from those previously described by others. Out of 149 strains isolated from cattle and sheep, 75 were typed and of the 25 strains obtained from other authors, only two. Generally more than 1 and up to 4 types occurred in a given animal. Nevertheless several common types occurred in sheep and cattle indicating that there was no host specificity.

When faecal samples from the four cows were examined at 0, 17 and 18 months there was no evidence that the change from winter to summer diet influenced either the numbers or types of *Strep. bovis* present although there was a change in type with passage of time. A similar change in type was observed by Emslie-Smith (1961) when he examined the *Escherichia coli* content of human faeces from one adult at frequent intervals over several months.

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References

Bailey, R. W. & Oxford, A. E. (1958). A quantitative study of the production of dextran from sucrose by rumen strains of *Streptococcus bovis*. *J. gen. Microbiol.* **19**, 130.

Barnes, E. M., Ingram, M. & Ingram, G. C. (1956). The distribution and significance of different species of faecal streptococci in bacon factories. *J. appl. Bact.* **19**, 204.

Breed, R. S., Murray, E. G. D. & Smith, N. R. (1957). Bergey's *Manual of Determinative Bacteriology*, 7th ed. London: Ballière, Tindall & Cox.

Dain, J. A., Neal, A. L. & Seeley, H. W. (1956). The effect of carbon dioxide on polysaccharide production by *Streptococcus bovis*. *J. Bact.* **72**, 209.

Dain, J. A., Seeley, H. W. & Neal, A. L. (1957). Production of hemolytic zones on blood agar by certain strains of *Streptococcus bovis* under increased CO_2 tension. *J. Bact.* **73**, 291.

Duguid, J. P. (1951). The demonstration of bacterial capsules and slime. *J. Path. Bact.* **63**, 673.

Elliott, S. D. (1957). Bacterial endocarditis of cattle. *Vet. Rec.* **69**, 1203.

Elliott, S. D. (1960). Type and group polysaccharides of group D streptococci. *J. exp. Med.* **111**, 621.

Emslie-Smith, A. H. (1961). Observations on the secular succession of types of *E. coli* and related organisms in the faecal flora of an adult human subject. *J. appl. Bact.* **24**, viii.

Hobson, P. N. & MacPherson, M. J. (1954). Some serological and chemical studies on materials extracted from an amylolytic streptococcus from the rumen of the sheep. *Biochem. J.* **57**, 145.

Lancefield, R. C. (1933). A serological differentiation of human and other groups of haemolytic streptococci. *J. exp. Med.* **57**, 571.

Lancefield, R. C. (1941). Specific relationship of cell composition to biological activity of haemolytic streptococci. *Harvey Lect.* Series **36**, 251.

MacPherson, M. J. (1953). Isolation and identification of amylolytic streptococci from the rumen of sheep. *J. Path. Bact.* **66**, 95.

Mann, S. O., Masson, F. M. M. & Oxford, A. E. (1954). Facultative anaerobic bacteria from the sheep's rumen. *J. gen. Microbiol.* **10**, 142.

Medrek, T. F. & Barnes, E. M. (1962). The distribution of group D streptococci in sheep and cattle. *J. appl. Bact.* **25**, 159.

Niven, C. F. (Jr.), Smiley, C. F. & Sherman, J. M. (1942). The hydrolysis of arginine by streptococci. *J. Bact.* **43**, 651.

Orla-Jensen, S. (1919). *The Lactic Acid Bacteria*. Copenhagen: A. F. Host & Son.

Perry, K. D. & Briggs, C. A. E. (1955). A common type antigen in streptococci of groups D and E. *J. Path. Bact.* **70**, 546.

Perry, K. D., Newland, L. G. M. & Briggs, C. A. E. (1958). Group-D streptococci with type antigens of group N. *J. Path. Bact.* **76**, 589.

Seeley, H. W. & Dain, J. A. (1960). Starch hydrolysing streptococci. *J. Bact.* **79**, 230.

Sharpe, M. E. & Fewins, B. G. (1960). Serological typing of strains of *Streptococcus faecium* and unclassified group D streptococci isolated from canned hams and pig intestines. *J. gen. Microbiol.* **23**, 621.

SHARPE, M. E. & SHATTOCK, P. M. F. (1952). The serological typing of group D streptococci associated with outbreaks of neonatal diarrhoea. *J. gen. Microbiol.* **6**, 150.

SHATTOCK, P. M. F. (1949). The streptococci of group D; the serological grouping of *Streptococcus bovis* and observations on serologically refractory strains. *J. gen. Microbiol.* **3**, 80.

SHERMAN, J. M. (1937). The streptococci. *Bact. Rev.* **1**, 3.

SHERMAN, J. M. (1938). The enterococci and related streptococci. *J. Bact.* **35**, 81.

SWIFT, H. F., WILSON, A. T. & LANCEFIELD, R. C. (1943). Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes. *J. exp. Med.* **78**, 127.

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KAESS, G. & WEIDEMANN, J. F. (1962). *J. appl. Bact.* **25** (2), 180–186.

AN APPARATUS FOR THE UNIFORM SPRAYING OF SOLID NUTRIENT SURFACES WITH BACTERIAL SUSPENSIONS

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SUMMARY: Slices of animal tissue or nutrient agar in Petri dishes can be sprayed with the apparatus described to obtain a uniformly distributed bacterial population. The standard error of duplicate counts of individual 1 cm² samples taken from different portions on sprayed areas was approximately 15%.

TO STUDY the influence of environmental conditions and applied physical or chemical treatments on the growth of micro-organisms on the surface of natural or artificial solid nutrients, it is generally desirable to be able to compare samples with definite initial populations uniformly distributed over the surface. A great variety of atomizers is used industrially for spray drying food, spraying pesticides to protect agricultural plantations, disintegrating fuel in burners, distributing aerosols, etc., and besides the design and the coefficients of discharge, it is mainly the pressure of the liquid before the nozzle which determines the size of the particles of spray (Fraser & Eisenklam, 1956). The atomizers are mostly designed to produce particles of a fairly uniform, definite size, rather than to give a uniform distribution of particles in a plane perpendicular to the axis of the spray cone. However, on the surface of solid nutrients on which the effect of different treatments has to be studied and from which definite areas have to be sampled to determine the number of surviving cells, the uniformity of the distribution of cells over the surface, which is inversely related to the amount of sampling required, is decisive. The equipment described below was designed to inoculate meat slices; but it may obviously also be used for, say, the inoculation of other tissues or nutrient agar in Petri dishes.

SPRAYING APPARATUS

The apparatus is shown diagrammatically in Figs. 1 and 2, and a photograph is presented in Plate 1. A flow of compressed air, maintained at a constant rate by the bleed cock BL and the Cartesian manostat CM, is supplied to the sprayer SP, the flow rate and pressure being observed by the rotameter R and the U-tube manometer U. The suspension of organisms to be sprayed is held at constant level in the jar CL by the supply in the Mariotte bottle MB. The suspension is aspirated through the hypodermic needle N by the air stream and is atomized into a fine spray. The spray deflector D directs the suspension into a container when no samples are being treated.

The samples to be sprayed are held in the cylindrical holder H behind the transparent plastic (methyl methacrylate) plate P and shutter F, which slides on the rods Ro. The shutter has a square aperture 10 cm × 10 cm and can be operated to expose a further aperture S, which is a sector of a circle centred on the middle of the holder. The shutter has three positions. At the beginning of the spraying of a sample the shutter is held in its upper position with the resting ledge RL_2 supported on the bolt B. The timing device T is started, and on the momentary closing of the first contact, K_1, the bolt is withdrawn against a spring by the electromagnet EM, allowing the shutter to fall. The bolt returns in time to intercept the shutter, holding it on ledge RL_1, thus exposing aperture S to the spray. A second contact (not shown) closes after a time, which can be adjusted from 0–60 sec, and again energizes EM. This causes bolt B to be retracted again, so that the shutter falls and rests on the bottom strip RL_3, thus screening the aperture from the spray.

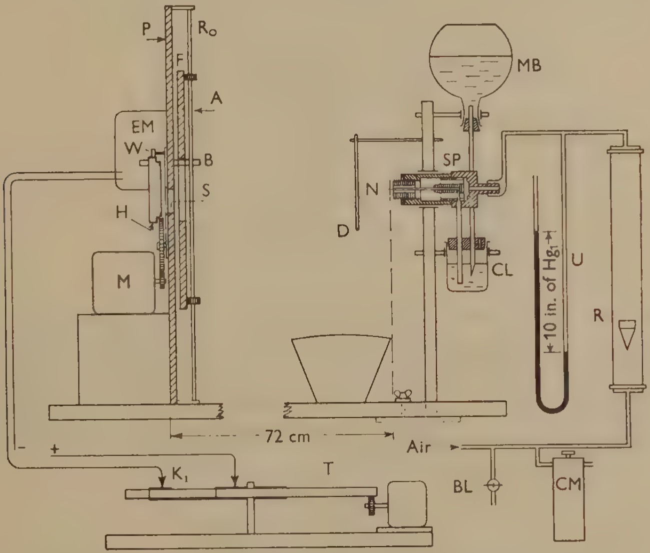

Fig. 1. Diagrammatic representation of spraying apparatus.

To obtain uniform distribution of spray over the sample the spray is directed to the centre of the aperture S, and can be accurately directed by hanging a mirror in a fixed position in front of the shutter. The mirror has a de-silvered spot corresponding to the desired centre of the spray, and the sprayer is adjusted on its stand so that the spray on the mirror is symmetrically distributed around the de-silvered spot. The holder H rests on three small rollers, W, and is rotated at 480 rev/min by the synchronous motor M through a train of gears.

To obtain a steady spray, the liquid entered the hypodermic needle N at almost atmospheric pressure, and passed through it in laminar flow. The 20 gauge needle

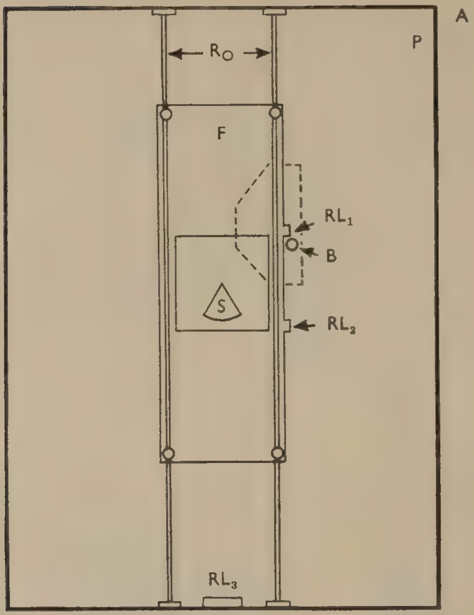

Fig. 2. Detail of spraying area, seen in direction A as shown in Fig. 1.

was centred in the air nozzle of 2 mm diameter. The disintegration of the liquid into fine droplets then depended only on the turbulence of the airflow. With a rate of flow of the air of 27 l/min and a pressure of 25 cm of Hg at the entrance to the nozzle, the cross section of the cone of spray at a distance of 72 cm was large compared with the area of the sample to be sprayed, so that a uniform distribution of spray on the sample could be expected.

EXPERIMENTS

In all work involving measurement of bacterial populations all vessels, instruments, and cutting and holding equipment were sterilized by heat. The surface of the muscle from which the samples were cut with a bacon slicer (sterilized with ethyl alcohol) was flamed with a Bunsen burner and all spray equipment was cleaned with ethyl alcohol.

Preliminary tests. In early trials the spray was used without rotating the sample, the spray being aimed at the centre of the sample. A different plate (P) was used which enabled samples 10 cm × 10 cm, or Petri dishes, to be exposed and sprayed when the shutter F was resting on the projection RL_1. With this arrangement, waxed papers 10 cm × 10 cm were sprayed for fixed intervals and the amount of water deposited was then determined by weighing. The variations within groups of ten papers sprayed in quick succession did not exceed 5%.

The extent of the fluctuation in the total population sprayed on to Petri dishes (exposed for 10 sec with plates stationary and the spray centred on the middle of the dish without the intercepting plate being used) was of the same order as the variation of the weight of water sprayed on paper. However, counts of organisms in individual squares of 1 cm side at distances of 0–1, 1–2 and 2–3 cm from the centre, as shown in the diagram (Fig. 3), showed that whereas there was no evidence of variation in the density of population within a distance of 2 cm from the centre

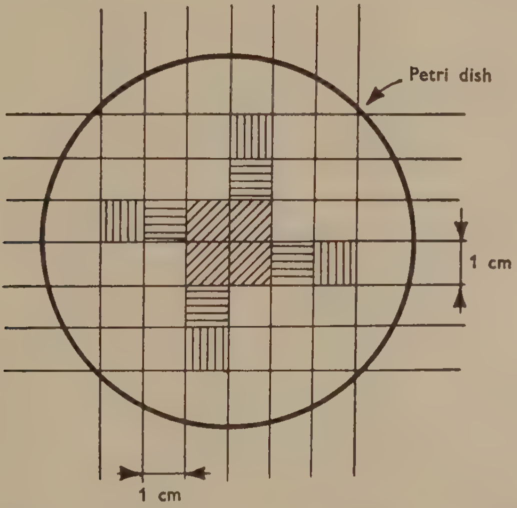

Fig. 3. Scheme of 1 cm² areas in Petri dishes, arranged in 0–1 cm (diagonal hatching), 1–2 cm (horizontal hatching), 2–3 cm (vertical hatching) distances from centre.

Table 1. *The analysis of variance of logarithms of counts of* 1 *cm² areas taken from* 1, 2 *and* 3 *cm distances from the centre of Petri dishes containing nutrient agar*

Source of variance	Degrees of freedom	Sum of squares	Mean square	Variance ratio F
Distances of 1 and 2 cm *v.* 3 cm	1	0·126774	0·126774	30·11*
Distance of 1 cm *v.* 2 cm	1	0·000415	0·000415	0·10
Distance	2	0·127189	0·063595	15·10*
Petri dishes	3	0·020891	0·006964	1·65
Distance × Petri dishes	6	0·047626	0·007938	1·89
Interreplicate†	36	0·151579	0·004211	
Totals	49	474474		

Sum of logarithms of 16 counts in 1 cm² areas at distance from centre of: 0–1 cm, 25·2661; 1–2 cm, 25·1553; 2–3 cm, 23·4664.

* Significant at a level of $P=0{\cdot}01$. † Quadruplicate.

of the dish, there was a significant decrease in the density of population as the distance increased beyond 2 cm (Table 1).

To correct asymmetry of the bacterial spray Ingram & Ingram (1955) rotated Petri dishes which contained meat slices and directed the spray at the centre of the dish.

In order to inoculate uniformly a circular area of 6·7 cm diameter, using the 4 cm diameter area of uniform spray only, the sample was rotated while exposed through the sector-shaped aperture S in plate P. The best use of the area of uniform spray is made when the sector angle is 72° and when the centre of the spray lies 2 cm away from the apex of the sector (which is at the centre of rotation of the sample). The sector is then the largest which can lie wholly within the area of uniform spray. As the circular sample is rotated past the sector it becomes uniformly covered. With a speed of rotation of the sample of 480 rev/min any heterogeneity of population density due to failure of an integral number of rotations to be made in the exposure period is small for exposures of not less than 10 sec. With the air flow used, the amount of water added to the sample in 10 sec (6–8 mg) has little effect on the moisture content of the surface of the sample, and this time was therefore normally used. Variation in count was obtained by changing the concentration of the bacterial suspension rather than changing the time of exposure.

Experiments with nutrient agar plates. For tests with the modified arrangement, *Pseudomonas* cultures were grown for 2 days at 20° in McEwan's peptone water to reduce precipitation of metabolic products, and the suspensions were filtered to prevent irregularity of flow in the spray needle. Petri dishes containing nutrient agar were sprayed with a suspension of this culture, using the spinning holder and sector aperture. The sprayed Petri dishes were incubated at 20° for 4 days. Quadruplicate counts were made of the numbers of organisms on squares of 1 cm side at distances of 0–1, 1–2 and 2–3 cm from the centre, as shown in the diagram (Fig. 3). An individual test normally involved ten dishes, giving 120 counts.

Experiments with meat slices. Musculus semitendinosus taken from a beef steer immediately after slaughter was used. Slices 1 mm thick were cut transverse to the length of the muscle with a sterile bacon slicer. The slices were mounted in stainless steel holders which exposed a circular area 6·7 cm in diameter. Four ml of a 2 day old bacterial culture (described above) were transferred to 500 ml of 0·5% peptone water and used as inoculum; this gave a concentration on the meat of about 10^3 organisms/cm^2 when one side of each slice was sprayed. Three slices were used in each experiment. Immediately after spraying. five circular samples of 1 cm^2 were cut from each slice at approximately equal distances from the centre of the slice, using a cutter which provided samples of exact area (Plate 1). During cutting, the unsprayed sterile, or nearly sterile, side of the meat slice rested on a sterile wooden cylinder of slightly greater diameter than the cutting cylinder.

Each 1 cm^2 sample was then homogenized for 1 min in 10 ml of 0·5% peptone water in a Buehler homogenizer (E. Buehler, Tübingen, W. Germany). Dilutions were made with 0·5% peptone water (Straka & Stokes, 1957), to minimize loss of viable cells. Plating on nutrient agar was done in quadruplicate, and the plates were incubated at 20° for 4 days.

RESULTS

Experiments with nutrient agar plates. Table 2 shows the analysis of variance of the logarithms of counts for a typical test, when a suspension of *Pseudomonas* was sprayed on rotated Petri dishes containing nutrient agar, using the sector-shaped aperture. There was no significant variation between dishes or from the centre to the outside of the dish.

Table 2. *The analysis of variance of the density of bacterial counts sprayed on nutrient agar in Petri dishes*

Source of variance	Degrees of freedom	Sum of squares	Mean square	Variance ratio F
Distances	2	0·023790	0·011895	1·129*
Petri dishes	9	0·145911	0·016212	1·539*
Distances × Petri dishes	18	0·280250	0·015569	1·478*
Interreplicate†	90	0·946961	0·010533	
Totals	119	1·396912		

* Not significant at a level of $P=0{\cdot}05$. † Quadruplicate.

Experiments with meat slices. The statistical analysis (logarithms of counts) of one typical experiment, given in Table 3, shows that plating errors contributed little to the overall variation, which was due largely to variation from point to point on any slice, possibly arising from turbulence in the spray. There is thus no point in using more than the normal duplicate platings. In this experiment the standard error (in terms of untransformed counts) of a single sample taken from a slice, and using duplicate platings, was 15%. For two other experiments the corresponding figures were 14·3% and 6·9%. In the first of these experiments, differences between estimates of the number of bacteria on single samples from the same slice would need to amount to 49% to be significant. For most work on measurements of growth rates of micro-organisms over a number of generations the above sensitivity would be adequate.

Table 3. *The analysis of variance of logarithms of counts from 1 cm² samples punched from sprayed meat slices*

Source of variance	Degrees of freedom	Mean square	Estimates of mean square*	Variance ratio F
Between slices	2	0·029027	$\sigma_1^2+4\sigma_2^2+20\sigma_3^2$	2·0†
Samples within slices	12	0·014496	$\sigma_1^2+4\sigma_2^2$	
Individual platings‡	45	0·001141	σ_1^2	

* $\sigma_1^2=0{\cdot}00114$; $\sigma_2^2=0{\cdot}00334$; $\sigma_3^2=0{\cdot}00073$.

† Not significant at a level of $P=0{\cdot}05$. ‡ Platings were done in quadruplicate.

The assistance of Mr. D. E. A. Plate and Mr. B. Mee in carrying out the microbiological tests is gratefully acknowledged.

REFERENCES

FRASER, R. P. & EISENKLAM, P. (1956). Liquid atomisation and the drop size of sprays. *Trans. Instn chem. Engrs, Lond.* **34**, 294.

INGRAM, M. & INGRAM, G. C. (1955). The growth of bacteria on horse muscle in relation to the changes after death leading to rigor mortis. *J. Sci. Fd Agric.* **6**, 602.

STRAKA, R. P. & STOKES, J. L. (1957). Rapid destruction of bacteria in commonly used diluents and its elimination. *Appl. Microbiol.* **5**, 21.

EXPLANATION OF PLATE

Photograph of device for cutting samples of meat from slices of about 1 mm thick; spare cutter heads and stainless steel frames for holding slices of meat are also shown.

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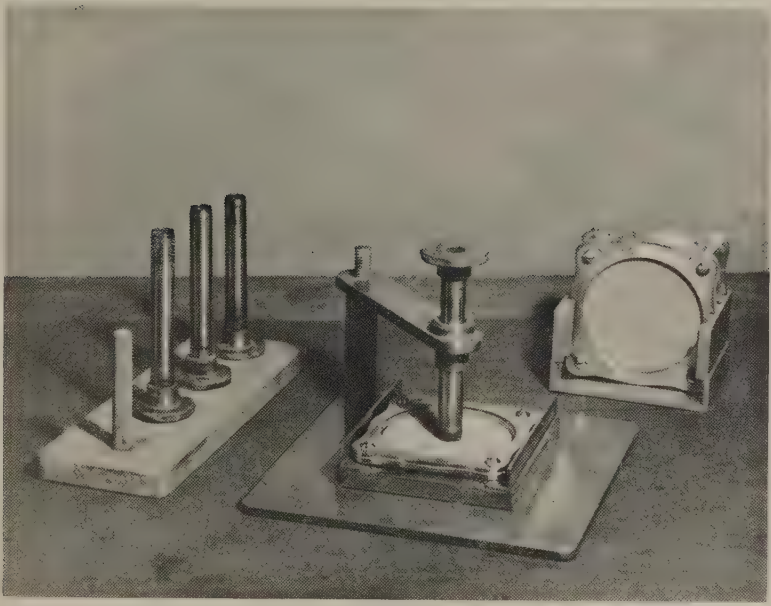

G. Kaess & J. F. Weidemann — An Apparatus for the Uniform Spraying of Solid Nutrient Surfaces with Bacterial Suspensions. Plate 1

Scherr, G. H. & Rafelson, M. E. (Jr.) (1962). *J. appl. Bact.* **25** (2), 187–194.

THE DIRECTED ISOLATION OF MUTANTS PRODUCING INCREASED AMOUNTS OF METABOLITES

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SUMMARY: A method is described whereby mutants having a directed increased yield of a metabolite may readily be isolated. A culture of bacterial cells is exposed to a gradient concentration of an antimetabolite that is inhibitory to the growth of the organism and mutants are isolated which survive because of an increased yield of the metabolite. This increased yield may be several times that formed by the parent culture. Techniques and results for increasing the yields of pyridoxine, nicotinic acid and thiamine are described.

Certain substances, usually similar in molecular structure to metabolites, can interfere with the normal utilization of metabolites and consequently cause the cessation of either metabolic activity or cell multiplication. Such substances are called antimetabolites, and their mode of action has been well documented by Martin (1951) and Wooley (1952). This interference is possible whether the metabolite is synthesized by the cells from other compounds or is a requirement which must be supplied *per se* to the culture medium. In general, however, the amount of antimetabolite necessary to inhibit is greater in the former instance than in the latter.

The specific requirements of organisms for essential metabolites have a genetic basis. Thus, under usual conditions of cell growth and multiplication, the progeny of cells that have a specific requirement for some chemical agent will also have this same requirement. The stability of genetic characteristics and their transfer from one generation to the next is occasionally marred by the occurrence of a mutant or variant which differs in some characteristic trait or traits from the parent cell from which it was derived. The significant feature of a mutant is that this altered trait is stable and is transferred thereafter to the succeeding generations, as was the original trait from which it differs.

In order to isolate a mutant from a microbial population, large numbers of organisms must usually be examined. Where the mutant has a character which enables it to survive under conditions which result in suppression of the parent type, then it is easily isolated. Bacteriophage resistant mutants afford an example. But the techniques hitherto reported for the isolation of biochemical mutants were not designed for the isolation of mutants which form increased amounts of specific metabolic products and whose selection from a population of organisms is thereby made feasible. The studies described here show how the employment of an antimetabolite which blocks the growth and multiplication of an organism through specific interference with the use of an essential metabolite makes feasible the isolation of mutants able to overcome the effect of the antimetabolite because they synthesize more of the metabolite than did the parent culture.

Since the advent of these studies, which were begun in 1956 (Scherr & Rafelson, 1957), Adelberg and his co-workers (Adelberg, 1958; Cohn & Adelberg, 1958) have reported similar findings with *Escherichia coli*, in which mutants resistant to thienylalanine, an antimetabolite of phenylalanine, were found to excrete large amounts of phenylalanine. The technique used by Adelberg was similar to that described here.

Adelberg also reported the isolation of mutants which excrete substances active against the following antimetabolites: serine methyl ester, *nor* valine, ethionine, *nor*-leucine, allylglycine, aspartophenone, α-hydrazino-*iso*-valeric acid, glutamyl hydrazide, diaminopurine, 5-methyl-tryptophan, methallyglycine, valine and α-hydrazino-*iso*-caproïc acid. Thus the strain resistant to ethionine has been shown to excrete methionine, and Adelberg pointed out that in each case the substance secreted by the mutant is presumably the normal metabolite corresponding to the analogue.

MATERIALS AND METHODS

General method. All attempts to isolate metabolic mutants were made on gradient plates in which the upper layer contained a suitable concentration of an antimetabolite.

Increased nicotinic acid production. A mutant with this ability was obtained from a culture of *E. coli* B/r, utilizing pyridine-3-sulphonic acid as the antimetabolite. A microbiological assay procedure was used, that of Barton-Wright (1952) and the organism was *Lactobacillus arabinosus*, which responds to nicotinic acid, nicotinamide and nicotinuric acid.

Appropriate dry samples of the *E. coli* cells were refluxed for 16 hr with petroleum ether to extract lipids which might interfere with the analysis. The extracted material (usually about 2 g) was suspended in 20 ml of 1 N HCl and hydrolyzed by autoclaving for 20 min at 15 lb/in^2. After cooling, 2·0 ml of 1 N sodium acetate solution were added, the pH adjusted to 4·5 with NaOH and the volume made up to an appropriate value. The solution was filtered, and an aliquot was adjusted to pH 6·8 and made up to an appropriate volume for assay. The standard curves obtained on the basal medium showed a linear portion and the results were calculated by the slope-ratio method.

Increased pyridoxine production. Saccharomyces microsporus NRRL Y-1550 and the blocking agent isoniazid were used to obtain a mutant giving enhanced pyridoxine production. To assay for pyridoxine, the cultures were thoroughly mixed, and 5·0 ml was removed from each, autoclaved at 121° for 5 min and cooled and centrifuged. Aliquots of the supernatants were assayed for pyridoxine in duplicate at two levels by the microbiological method using *Saccharomyces carlsbergensis*

NRRL Y-1089 as described in Assay Methods (1951), except that the assays were carried out in 125 ml Ehrlenmeyer flasks instead of test tubes and the flasks were shaken for 18 hr on a model no. 10 Brunswick Gyrotory Shaker at 250 rev/min.

The 45 ml of culture remaining in each flask after removal of the aliquot for pyridoxine assay was centrifuged, the cell pellet washed once with sterile buffered saline, recentrifuged and the residue transferred to a tared weighing dish. The cells were then dried overnight at 105°, cooled in a desiccator over anhydrous $CaCl_2$ for 1 hr and weighed.

Increased thiamine production. E. coli B/r (ATCC 12407, N12-17) and the antimetabolite pyrithiamine HBr were used to obtain mutants giving increased yields of thiamine. The sample to be assayed consisted of the washed cells from the entire 50 ml of culture and its preparation for assay was carried out as described in Assay Methods (1951), the essential features being: that the sample is suspended in 50 ml of 0·1 N HCl and autoclaved for 15 min at 15 lb/in^2; the acid extract of the cell sample is cooled and filtered and 5 ml of a thiamine free phosphatase preparation added; after incubation at 45° and pH 4·5 for 3 hr the extract is cooled and filtered, adjusted to pH 3·5 and passed through a column of Decalso; the column is then washed three times with 30 ml of water at 70° and eluted with 50 ml of acidified KCl solution; an aliquot of the acidified KCl eluate is then converted to thiochrome which is extracted and the fluorescence measured. A Coleman Model 12-A Photofluorometer was used. Standard thiamine solutions were similarly treated and yielded the following results: a standard of 10·0 μg gave, in duplicate determinations, 9·7 and 10·1 μg, mean 9·9; a standard of 20·0 μg gave, in duplicate determinations, 19·8 and 19·5 μg, mean 19·7; and a standard of 40·0 μg gave, in duplicate determinations, 41·2 and 39·4 μg, mean 40·3 μg.

Experiments and Results

Experiments with the nicotinic acid antagonist pyridine-3-sulphonic acid

Isolation of a resistant mutant. A gradient plate was poured in which the lower layer consisted of the following medium: $(NH_4)_2SO_4$, 1·5 g; K_2HPO_4, 1·5 g; KH_2PO_4, 1·5 g; $MgSO_4.7H_2O$, 0·6 g; NaCl, 0·013 g; $FeSO_4.H_2O$, 0·02 g; $MnSO_4.2H_2O$, 0·913 g; glucose, 3·0 g; biotin, 0·1 μg/ml; distilled water, 150 ml; agar, 2·25 g. The top layer contained the same medium plus 0·01 M pyridine-3-sulphonic acid. A heavy inoculum of *E. coli* B/r was streaked over the surface of the pyridine-3-sulphonic acid gradient plate, and after incubation at 37° for 48 hr a confluent growth was seen up to the point at which there was an inhibiting concentration of the antimetabolite. Beyond this point there were isolated colonies. These presumably consisted of mutants having the ability to multiply in the presence of concentrations of the antimetabolite inhibitory to the parent culture. One of these colonies was inoculated on to a slant of the above agar medium not containing the antimetabolite and serially subcultured three times to fresh similar slants. This culture, designated 5-218D, was then restreaked on the surface of another gradient plate similar to the first, and after incubation for 48 hr confluent growth was observed over the whole plate. The culture 5-218D was then serially transferred several times on slants of the medium without antimetabolite and finally inoculated into 1 litre of the medium in liquid form and incubated at 37°. After 7 days the cells were harvested by centrifugation for 30 min at 2,000 rev/min and 0° and both the cells and supernatant were separately assayed for nicotinic acid.

Nicotinic acid production by parent and mutant. In order to compare the production of nicotinic acid by culture 5-218D with that of the parent, and to determine the degree of variation of the nicotinic acid assay, three clone isolates of the parent culture B/r obtained as follows were examined.

The culture of *E. coli* B/r was streaked out on the agar medium without the antimetabolite and incubated at 37° for approximately 96 hr. Then three isolated colonies (designated by the code letters 5-211W, 5-211X, and 5-211Y) were transferred to slants of the same medium. These three isolates were then inoculated into flasks containing 1,000 ml of the liquid medium and incubated at 37° for approximately 7 days (and 3 hr). The cells were harvested by centrifugation for 30 min at 2,000 rev/min and 0° and both the cells and supernatant were separately assayed for nicotinic acid.

The nicotinic acid contents of the culture supernatants of mutant 5-218D and the three parent isolates 5-211W, 5-211X and 5-211Y were essentially equal to that of the uninoculated medium, and only the summarized results of assays done on the cells of these cultures are given in Table 1.

Table 1. *The results of nicotinic acid assays done on the cells from cultures of* E. coli *B/r and a mutant thereof resistant to pyridine-3-sulphonic acid*

Code no. and type of *E. coli* B/r	Nicotinic acid formed* (μg/g dry cells)
5-211W (parent)	68·8
5-211X (parent)	65·1
5-211Y (parent)	75·1
5-218D (mutant)	265·0

* Means of 6 determinations.

The variability of the assay results for the three parent isolates are within the experimental error of the assay method.

It is clear that culture 5-218D was a stable variant showing a marked increase in production of nicotinic acid or derivatives thereof. In order to be sure that contamination with organisms other than *E. coli* was not responsible for differences between strain 5-218D and the three parent type isolates 5-211W, 5-211X and 5-211Y, a number of accepted identifying tests were applied, and it proved that all those isolates and the original culture *E. coli* B/r from which they were derived gave + + − − IMViC reactions, fermented lactose with the production of acid and gas in 24 hr at 37°, were Gram-negative and had the characteristic colonial morphology on eosin-methylene blue agar.

Experiments with the pyridoxine antagonist isoniazid

Isolation of a resistant mutant. Slants of *Saccharomyces microsporus* NRRL Y-1550 were maintained on agar slants of a medium, arbitrarily designated medium A, which contained: $(NH_4)_2SO_4$, 0·75 g; K_2HPO_4, 0·75 g; KH_2PO_4, 0·75 g; $MgSO_4.7H_2O$, 0·30 g; NaCl, 0·015 g; $FeSO_4.7H_2O$, 0·01 g; $MnSO_4.2H_2O$, 0·0065 g; glucose, 1·5 g; biotin, 0·1 μg/ml; distilled water, 150 ml; agar, 2·25 g.

Cells from a 48 hr slant culture of *Sacch. microsporus* NRRL Y-1550 were suspended in sterile buffered saline (NaH_2PO_4, 0·57 g; Na_2HPO_4, 2·5 g; NaCl, 8·5 g; distilled water, 1,000 ml) and inoculated on to a gradient plate containing medium A, the upper layer of which contained isoniazid at the concentration of 2 g/100 ml of medium.

After incubation for approximately 2 days at 26°, growth was seen to extend for 35 mm across the gradient plate. Beyond this there were isolated colonies which were resistant to the isoniazid. Several of these were picked, transferred to agar slants of medium A and incubated at 26° for 2 days. One of these cultures, designated *Sacch. microsporus* 6-191 I, was again streaked on a gradient plate similar to the first, and after incubation growth was seen to extend for 81 mm across the gradient, indicating that this mutant was able to grow in the presence of a concentration of the antimetabolite which completely inhibited its parent.

Pyridoxine production by parent and mutant. Cells from a 48 hr agar slant culture of *Sacch. microsporus* NRRL Y-1500 were suspended in sterile buffered saline, centrifuged, resuspended in 10 ml of sterile buffered saline and 1 ml of the suspension added to each of three 250 ml Ehrlenmeyer flasks containing 50 ml of pyridoxine free basal single strength medium, the composition of which was that given for pyridoxine assay in Assay Methods (1951) except that the glucose content was reduced to 3·0% and the pH adjusted to 6·0. Cells from a 48 hr culture of the mutant *Sacch. microsporus* 6-191 I were similarly treated.

Thus six flasks in all were prepared, three of the yeast parent labelled A, B and C and three of the mutant 6-191 I labelled D, E and F. The flasks were then incubated at 26° on a model no. 10 New Brunswick Gyrotory Shaker at 250 rev/min, and after

Table 2. *The production of pyridoxine by parent and isoniazid resistant mutant cultures of* Sacch. microsporus

No. and type of culture	Age of culture (days)	Yield of dry cells (mg/ml)	Pyridoxine yields in	
			Dry cells (mμg/mg)	Supernatant* (mμg/ml)
A (parent)	4	9·24	43·79	404·7
D (mutant)	4	8·54	320·0	2,750·0
B (parent)	7	7·10	51·4	365·0
C (parent)	7	8·66	43·8	379·2
E (mutant)	7	7·49	375·9	2,816·6†
F (mutant)	7	7·56	377·9	2,857·5

* Means of 4 determinations. † Mean of 3 determinations only.

4 days the cultures 26-19A and 26-19D were removed for pyridoxine and dry cell determinations. Cultures 26-19B, C, E and F were removed after 7 days. Before the assays sufficient distilled water was added to each culture to restore it to the original volume.

Table 2 shows, for the parent and mutant cultures, the dry weights of cells produced, their pyridoxine contents, and the amounts of pyridoxine excreted into the medium. The significant point is the large difference in the yields given by the mutant culture 6-191 I and the parent culture from which it was derived, but it is of interest to note that the pyrodixine contents of the dry cells changed little between the 4th and 7th days.

It is also clear that the ability of this mutant to secrete pyridoxine in large amounts does not depend upon its being grown on or in a medium containing the inhibitor which made possible its isolation. That the mutant character was stable was evident from the fact that when *Sacch. microsporus* 6-191 I was re-examined 1 year after its isolation, having been kept meanwhile on isoniazid free medium, it still retained its enhanced ability to form pyridoxine.

Experiments with the thiamine antagonist pyrithiamine

Isolation of resistant mutants. E. coli B/r (ATCC 12407, N 12-17) was serially transferred twice on slants of a medium, arbitrarily designated medium C, which contained: glucose, 4·0 g; KH_2PO_4, 3·0 g; NH_4Cl, 1·0 g; $Na_2HPO_4.12H_2O$, 15·0 g; NaCl, 0·5 g; $MgSO_4.7H_2O$, 0·2 g; distilled water, 1,000 ml; agar, 15 g.

The second transfer was used to inoculate a wide mouth 500 ml Ehrlenmeyer flask containing 50 ml of medium C to which had been added 0·2% (w/v) of Oxoid yeast extract. This was then incubated at 37° on a New Brunswick Gyrotory Shaker model S-3 at 250 rev/min for 48 hr, when 25 ml of the culture were centrifuged and washed once with sterile saline buffered with phosphates to pH 7·0. The cells were then resuspended in 4·0 ml of an irradiation medium. This contained: adenine sulphate, 1·0 mg; cytosine, 1·0 mg; guanine HCl, 1·0 mg; thymine, 1·0 mg; uracil, 1·0 mg; yeast extract (Oxoid), 200 mg; distilled water, 100 ml. It was sterilized in the autoclave for 15 min at 15 lb/in^2.

A 2·4 ml quantity of the suspension of cells in the irradiation medium was placed in a 125 ml round bottom quartz flask and exposed to ultraviolet light from an 8 W germicidal lamp for 5 min at a distance of 6 in. while being slowly rotated. The contents of the quartz flask were then transferred to a 250 ml Ehrlenmeyer flask containing 25 ml of a medium, designated medium D, which contained: glucose, 1·0 g; enzymic digest of casein, 2·0 g; pyrithiamine HBr, 300 μg; distilled water, 100 ml. The resulting suspension was then placed on the rotary shaker at 37° and exposed for 25 min to the light from two General Electric photoflood lamps. The shaking, without floodlights, was continued for another 2 hr. The culture was then centrifuged, the cells washed once with sterile phosphate buffered saline and resuspended in 10 ml of sterile buffered saline, and 1 ml of this suspension was then spread over the surface of a gradient plate of medium C, with the upper layer containing 50 μg of pyrithiamine HBr/ml. After the inoculated gradient plate had been incubated at 37° for 48 hr heavy growth appeared at the end of the plate where the concentration of pyrithiamine HBr was the lowest. The density of growth gradually diminished toward the opposite side of the plate and at the extreme end, where the concentration of the inhibitor was the greatest, isolated colonies appeared which were considered to be mutants. Several of these were transferred to agar slants of medium C and incubated at 37° for 48 hr.

Thiamine production by parent and mutants. Five strains of *E. coli* B/r which tolerated pyrithiamine at 3 μg/ml were compared with the parent for their ability to synthesize thiamine. Portions of 50 ml of medium C without agar, contained in wide mouth 500 ml Ehrlenmeyer flasks, were inoculated in duplicate with the parent and presumed mutants. These shake flask cultures were incubated at 37° for 48 hr on the New Brunswick Gyrotory shaker at 250 rev/min, when the cells were removed by centrifugation, washed, lyophilized and a weighed sample taken for analysis. The results of the examination of duplicate cultures are given in Table 3.

Table 3. *The production of thiamine by* E. coli *B/r* (ATCC 12407, N 12-17) *and pyrithiamine resistant mutants derived from it*

Culture no. and type		Thiamine content (μg/g dry cells)		
		1	2	Mean
N 12-17	Parent	16·2	17·0	16·6
29-296 A	Mutant	43·6	50·2	46·9
29-296 B	Mutant	20·3	18·9	19·6
29-296 C	Mutant	52·7*	56·4	54·5
29-296 D	Mutant	33·9	29·8	31·8
29-296 G	Mutant	40·5	38·3	39·4

* When 20 μg of thiamine were added to a sample of this culture a value of 74·6 was obtained, indicating the accuracy of determination.

Only one of the pyrithiamine resistant cultures yielded essentially the same amount of thiamine as the parent: all the others formed two or three times as much.

DISCUSSION

There are reasons other than an increased yield of a specific metabolite that could explain the survival of a mutant in the presence of an inhibitory concentration of an antimetabolite. For example, if a mutant arose which did not make or require the metabolite at all, then the role of the antimetabolite in inhibiting multiplication would be negated. However, judging from the ease with which mutant cultures with increased capacity for synthesis of a metabolite were isolated in populations of cells surviving the inhibitory concentrations of the antimetabolites, many of the organisms that survived must have been of that type.

It is notable that in all the studies so far performed the increment in yield produced by the mutant over that found in the parent was large. We are normally accustomed to thinking in terms of 10%, 20% or possibly 50% increase in yield of metabolites by mutants. However, the yields of metabolites by the mutants isolated in these studies represented three- to fivefold increases over those given by the parent type. These yields are extremely high and underline the value of the method used.

The methods described here utilizing gradient plates can be equated to a serial dilution technique in which the parent organism is inoculated into a basal medium containing a minimal concentration of the antimetabolite and then, after suitable incubation, serially transferred through increasing concentrations of the antimetabolite until the final culture consists of cells which are presumably mutants having the increased production of metabolite.

Experience has shown that with both the gradient plate technique and the tube dilution method, there is some advantage in picking the presumed mutants to a medium still containing a concentration of the antimetabolite inhibitory to the parent type. This served in many instances to purify genetically a clone that had in it a few genetic organelles of the parent type.

Although the principal studies performed by the authors have been concerned with bacteria and yeasts, the technique appears applicable to other micro-organisms or even to cultures of cells of higher plants that can be grown in large quantities and subjected to the same conditions of growth and isolation as those described here. In fact, the techniques of Puck and his colleagues for the clonal growth of mammalian cells *in vitro* might well be applicable for the enhanced production of unique biological substances from tissue culture systems.

REFERENCES

ADELBERG, E. A. (1958). Selection of bacterial mutants which excrete antagonists of antimetabolites. *J. Bact.* **76**, 326.

ASSAY METHODS (1951). *Methods of Vitamin Assay*, 2nd ed. Edited by the Association of Vitamin Chemists, Inc. New York: Interscience Publ., Inc.

BARTON-WRIGHT, E. C. (1952). *Microbiological Assay of the Vitamin B Complex and Amino Acids.* New York: Pitman Publ. Corp.

COHN, G. N. & ADELBERG, E. A. (1958). Kinetics of incorporation of *p*-fluorophenylalanine by a mutant of *Escherichia coli* resistant to this analogue. *J. Bact.* **76**, 328.

MARTIN, G. J. (1951). *Biological Antagonism.* New York: Blakiston Co.

SCHERR, G. H. & RAFELSON, M. E. (Jr.) (1957). Method of increasing metabolic product yield of organism. U.S. Patent no. 662,004, filed May 28, 1957.

WOOLEY, D. W. (1952). *A Study of Antimetabolites.* New York: John Wiley & Son.

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DAVIS, J. G. (1962). *J. appl. Bact.* **25** (2), 195–201.

IODOPHORS AS DETERGENT-STERILIZERS

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1. INTRODUCTION

IODINE as an antiseptic has a history longer than that of microbiology as a science, but certain of its properties militate against its wide adoption as a disinfectant or sterilizing agent. Thus it is only sparingly soluble in cold water (1 part in 3,450 or 290 p/m at 20°), although much more soluble in hot water (1 part in 250 or 4,000 p/m at 100°). Stronger solutions can be made in aqueous alcohol (tincture) or in potassium iodide solutions, but this increases the expense. Iodine solutions have an odour and stain fabrics, tissues and most organic matter, and from the dairy and other food industry aspects the fact that iodine can corrode some metals is a disadvantage, although it is not so reactive as chlorine.

The discovery by Shelanski that polyvinyl pyrrolidone and other surface active substances could solubilize iodine to form compounds that retained its desirable germicidal activity but not its undesirable properties, brought about a revolution in the use of iodine for sanitizing purposes. He named these new substances iodophors (iodine bearers). In solution they are able to solubilize up to nearly 30% of their weight of iodine, of which from 70–80% may be released as 'available iodine' when the concentrated solution is diluted. Although their constitution is not known exactly, it is clear that the iodine is bound in the form of micellar aggregates and that on dilution the micelles are dispersed and the linkage of the iodine is progressively weakened. At dilutions greater than the minimum micellar concentration the solution can be regarded as a simple aqueous solution of iodine. Some of the iodine (20–30%) may ultimately be converted to inorganic iodide, but this is not a serious handicap in view of the powerful germicidal properties of free iodine.

The surface active substances may be non-ionic, anionic or cationic (Darragh & Johnson, 1952; Rohm & Haas Co., 1954; Cantor, Most & Shelanski, 1956; Moore & Hardwick, 1956). The last two kinds are affected appreciably by pH value and the hardness of the water, and non-ionic compounds are therefore the best. However, germicidal power and milk stone removal are favoured by low pH values, and high pH values lead to iodide formation. It is therefore standard practice to add a little acid to the preparation. Phosphoric acid is commonly used to avoid corrosion, but fundamentally any acid would serve. Hydrochloric and various organic acids have all been used. In addition to its anticorrosive properties, phosphoric acid exerts a considerable buffering effect in the pH range of use, and so is probably the best single acid for the purpose. Mixtures of acids can be used.

Most attention has been given to non-ionic surface active substances prepared by ethoxylation. The most efficient of these can absorb from 25–28% of iodine (Bartlett & Schmidt, 1957). In general the alkylphenoxypolyglycol ethers have proved to be the most suitable of the non-ionic surface active agents.

2. Bactericidal Power

As the available iodine in use solutions (from 12·5–50 p/m) of iodophors is in simple aqueous solution, all data on the bactericidal power of iodine apply equally to equivalent solutions of iodophors (Mueller, 1955).

Using a modification of the capacity test of Cantor & Shelanski (1951), Ortenzio (1955) found that whereas a strength of 25 p/m of available iodine was equivalent to 200 p/m of available chlorine, 12·5 and 6·25 p/m of iodine were slightly inferior to 100 and 50 p/m of chlorine, respectively.

Chambers *et al.* (1952) found that under the favourable conditions of pH 6·5 and 20–26° the enterobacteria tested were all killed in 1 min by 0·6 p/m. Under the adverse conditions of pH 9·15 and 2–5°, a concentration of 4·3 p/m was required. Lawrence, Carpenter & Naylor-Foote (1957) have published data on bacteria and fungi. Using the silk loop method of Ortenzio, Stuart & Friedl (1953), they found that 1,000 p/m destroyed *Bacillus subtilis* spores in 6–10 hr, and 800 and 500 p/m in 10–24 hr. *Mycobacterium tuberculosis* was killed by 50 p/m in 2–10 min, and pathogenic fungi by 40–100 p/m in 10 min. Iodine is also effective against viruses (Gershenfeld, 1955*a*,*b*,*c*). Similar data for the bactericidal, fungicidal and viricidal activities of iodophors have been published by Bartlett & Schmidt (1957).

It may be noted that iodine has a much higher atomic weight (127) than chlorine (35·5), and that quaternary ammonium compounds (QACs) have molecular weights in the region of 300. It may be stated that on the p/m basis iodine is about 8 times as effective a germicide as chlorine (Gershenfeld & Witlin, 1955), which is itself much more effective than the QACs. On a molar weight basis the ratio of effectiveness of iodine in relation to chlorine becomes about 30:1, and the ratio is still high for chlorine in relation to QACs. As chlorine is chemically more reactive than iodine, it is evident that there must be some special effect, other than a simple oxidizing effect, which is responsible for the superiority of the iodine molecule (or atom) over the chlorine molecule (or atom). Possibly the form of the molecule, or a 'persistence effect' may be responsible. Chlorine may be rapidly and firmly bound in the protoplasm, and only a small part may interfere with vital processes. Iodine may be only loosely bound and so relatively more available for intra-protoplasmic transfer, and iodine in the bacterial protoplasm may behave as it does in the iodophor itself.

3. FACTORS AFFECTING THEIR USE

The first applications of iodophors in the dairy industry were by Lazarus (1952, 1954), Johns (1954) and Johns & Berzins (1954), in the U.S.A. and Canada.

Data on the sanitization of various materials by iodophors, hypochlorite and QACs have been given by Hays, Burroughs & Johns (1958).

The effect of pH value on germicidal powers. Like chlorine, iodine is the more effective as a germicide the lower the pH value, but it appears to be less affected by acidity than chlorine. The bactericidal power of hypochlorite is associated with the content of hypochlorous acid, whereas that of iodine appears to be due to elemental iodine. Broadly speaking, we may say that the germicidal power of chlorine rises steeply with falling pH, while that of QACs rises with rising pH. Iodine is less sensitive to variations in pH value than either.

The effect of soiling material. Mueller (1955) found that a strength of 12·5–25 p/m of available iodine was roughly equivalent to 100 p/m of available chlorine against *Escherichia coli*, *Salmonella typhosa*, *Staphylococcus aureus* and *Pseudomonas pyocyanea* in hard water and in the presence of 1% of dish wash soil and 1% of milk.

Iodophor solutions maintain very good killing powers in the presence of organic matter as long as the pH value does not rise above 4. Germicidal power falls off above this value and is much less at pH 7.

4. ADVANTAGES AND DISADVANTAGES OF IODOPHORS

(a) *Advantages*

The advantages are numerous, and may be listed as follows.

1. They permit the application of iodine, a powerful germicide, in a safe and convenient way.

2. They are almost odourless.

3. They are almost nonirritant (Cantor, Most & Shelanski, 1956) and do not sensitize humans.

4. Their toxicity is very low (Cantor, Most & Shelanski, 1956).

5. Iodophors are not only powerful germicides; they are also powerful detergents. Being surface active they possess strong wetting powers and penetrate 'dirt'. Although they may not dissolve protein as readily as alkaline detergents, they at least compensate for this by their powerful disintegrating action on milk stone. It is well recognized that even with the most effective alkaline detergents or detergent-sanitizers there is a steady build-up of milk stone unless certain precautions are observed. Processing dairies normally use an acid treatment at regular intervals; farm dairies frequently fail to do this and milk stone can be found on much of the milk equipment. On first using an iodophor staining of the equipment is often

evident, but this disappears after two or three applications of the iodophor. The same product can be used both as a detergent and as a sterilizing agent, although in practice the two actions are intermingled. The concentration for the former is commonly twice that for the latter use, e.g. 25 and 12·5 p/m of available iodine.

6. The germicidal activity of an iodophor is simply that of the free iodine present. The properties of iodine as a germicide have long been recognized. It is almost equally powerful against all vegetative types of bacteria (McCulloch, 1945).

7. Iodine exerts its germicidal action over a wide pH range. Although activity is reduced by rising pH, iodophors can be used at much lower pH values than hypochlorite solutions which evolve chlorine gas below pH 5. Thus iodophors are not so limited in their application as hypochlorites (activity reduced by alkalinity) and QACs (activity reduced by acidity). Some of the latter are greatly affected by water hardness.

8. They have low temperature coefficients, and may still be one-quarter as powerful at 2–5° as at 20–26° (Chambers *et al.*, 1952).

9. Although affected by organic matter, like all sterilizing agents, iodine is less affected than most as shown by capacity tests in which successive amounts of bacterial suspension are added to the sterilizing solution. Iodine has been found to be superior in this respect to chlorine, which in turn is superior to QACs (Gershenfeld & Witlin, 1955, 1958).

10. Organisms do not develop resistance to iodine.

11. When the iodine has all been absorbed by organic matter (just as chlorine is absorbed), the yellow-brown colour disappears. The residual iodine is then reduced to less than 6–8 p/m and the solution should be replaced. The colour of the iodophor solution thus acts as an automatic indicator of potency.

12. Iodophors stain soiling matter such as milk solids, milk stone, pus and blood, and so automatically indicate if equipment is physically clean. They do not stain metals, as reported by some users, who apparently have never succeeded in cleaning their equipment properly. Although potentially more corrosive than alkaline detergents, iodophor solutions are not corrosive if properly used, i.e. at the right concentrations for the right time at the prescribed temperature, and with adequate rinsing. Silver is the only susceptible metal.

13. Iodophor preparations are stable in storage.

(b) *Disadvantages*

The relatively few disadvantages of iodophors are as follows.

1. If used at higher concentrations than necessary they may be more expensive than ordinary hypochlorites and some other chlorine compounds, although not so expensive as some QACs and other germicides.

2. If used negligently (e.g. in excessive concentrations, and failing to discard the solution and rinse the utensil) iodophors may be more corrosive than alkaline detergents based on QACs.

3. Complaints of odour and staining of the hands have been received on isolated occasions. Investigation indicated that these arose from incorrect methods of use, e.g. with very hot water.

5. APPLICATIONS IN DAIRYING

Investigations with dairy equipment. In the only investigation of iodophors previously reported in the U.K., Cousins, Hoy & Clegg (1959) compared two iodophors with a hypochlorite, using suspension tests (Cousins, 1951), the Hoy can test (Hoy & Clegg, 1953) and water sterilizing tests (Cousins & Clegg, 1956). In general these workers confirmed previous findings on the efficiency of iodophors and the non-selectivity of its germicidal powers. A proprietary iodophor (buffered with phosphoric acid) was about 3 times as effective as hypochlorite in a suspension test with 10% of milk. Using the Hoy can test, however, the recommended use strength of the iodophor (25 p/m) was inferior to hypochlorite at 300 p/m. The figures in this paper indicated that, to compare with 300 p/m of chlorine, used in conjunction with 0·25% of sodium carbonate, the iodophor would have to be used at about 100 p/m.

An iodophor without acid was found to be markedly inferior to the buffered acid iodophor. The water sterilizing tests showed that the iodophor was not quite as efficient as hypochlorite, 20 p/m of the latter being equivalent to about 25 p/m of the former.

The difference between the results of the suspension and of the can or surface film tests is of particular significance for the dairy industry. There can be little doubt of the correctness of the explanation offered by Cousins, Hoy & Clegg (1959), namely that in a surface film test the detergent aspect of the product dominates the behaviour, and so the result of the test.

Huurnink (1959) was able to clean and sterilize a 10,000 litre tank by spraying with an iodophor solution for 20 min. The only criticisms offered by this worker were on the basis of cost and temperature sensitivity (but with respect to the latter see section 4, (a) 8).

In Finland, Peltola & Mälkki (1959*a*,*b*) have found two iodophors to be as effective as hypochlorite for sanitizing equipment. They also found that iodophors were less sensitive than chlorine to milk residues. The iodophors were not corrosive to aluminium.

Corrosion effects. Johns (1954) studied this aspect and found that corrosion of stainless steel was negligible. The effect on tinned steel was about the same as that of an alkaline hypochlorite and much less than that of an acid chlorine compound.

The effect of iodophor residues on milk. Although all traces of detergents and sterilizing agents should always be rinsed away, there is always the possibility that traces may get into milk. Johns (1954) found that amounts up to 10 p/m of iodine imparted no off-flavour to milk, either before or after pasteurization.

Mastitis control. The iodophors have been found to be valuable in mastitis control work. They are especially efficient for udder washing and for the treatment of clusters.

The dipping of teats in an iodophor solution (1% available iodine) immediately after milking, combined with pasteurization (165–170° F for 15 sec) of the teat cups, has been found to reduce significantly the incidence of haemolytic staphylococci on teats and inflations (Newbould & Barnum, 1956, 1958, 1960).

Iodophors as self-indicating sterilizing agents. On account of the well recognized effect of organic matter on the efficiency of a germicide (see Davis, 1961), the built-in indicator of iodophors is a particularly helpful feature when organic matter is present. Other germicides may become neutralized without the fact being realized by the user.

6. REFERENCES

BARTLETT, P. G. & SCHMIDT, W. (1957). Surfactant-iodine complexes as germicides. *Appl. Microbiol.* **5**, 355.

CANTOR, A. & SHELANSKI, H. A. (1951). A capacity test for germicidal action. *Soap, N.Y.*, **27** (2), 133.

CANTOR, A., MOST, S. & SHELANSKI, M. (1956). Iodophor-iodine shampoos. *J. Soc. cosmet. Chem.* **7**, 419.

CHAMBERS, C. W., KABLER, P. W., MALONEY, G. & BRYANT, A. (1952). Iodine as a bactericide. *Soap, N.Y.*, **28** (10), 149.

COUSINS, C. M. (1951). A preliminary bacteriological screening test for quaternary ammonium compounds and formulations. *Proc. Soc. appl. Bact.* **14**, 184.

COUSINS, C. M. & CLEGG, L. F. L. (1956). The effect of water hardness and temperature on water sterilization by mixtures of detergents and quaternary ammonium compounds. *J. appl. Bact.* **19**, 250.

COUSINS, C. M., HOY, W. A. & CLEGG, L. F. L. (1959). The evaluation of the suitability of iodine-bearing detergent-sterilizers for farm purposes. *Int. Dairy Congr., London*, **3**, 1807.

DARRAGH, J. L. & JOHNSON, G. B. (1952). Germicidal detergent compositions. U.S. Pat. no. 2,746,928.

DAVIS, J. G. (1961). Methods for the evaluation of the antibacterial activity of surface active compounds: technical aspects of the problem. *J. appl. Bact.* **23**, 318.

GERSHENFELD, L. (1955*a*). Iodine as a germicide. *Soap Chem. Spec.* **31** (4), 139.

GERSHENFELD, L. (1955b). Iodine and sanitation. *J. Milk Tech.* **18**, 220.

GERSHENFELD, L. (1955*c*). Sanitizer-detergents. *Soap Chem. Spec.* **31** (5), 164; (6), 163.

GERSHENFELD, L. & WITLIN, B. (1955). Iodine sanitizing solutions. *Soap Chem. Spec.* **31** (12), 189.

GERSHENFELD, L. & WITLIN, B. (1958). Iodine for quick acting sanitizers. *Soap Chem. Spec.* **34** (7), 67.

HAYS, G. L., BURROUGHS, J. D. & JOHNS, D. H. (1958). The cleanability of materials in contact with dairy products. *J. Milk Tech.* **21**, 68.

HOY, W. A. & CLEGG, L. F. L. (1953). Testing dairy farm detergent-sterilizers using soiled ten gallon cans. *Proc. Soc. appl. Bact.* **16**, i.

HUURNINK, J. (1959). The cleaning and disinfection of tanks with the aid of iodophors. *Misset's Zuivel.* **65**, 1072.

JOHNS, C. K. (1954). Iodophors as sanitizing agents. *Canad. J. Technol.* **32**, 71.

JOHNS, C. K. & BERZINS, I. (1954). Iodophors as preservatives for milk. *J. Milk Tech.* **17**, 313.

LAWRENCE, C. A., CARPENTER, C. M. & NAYLOR-FOOTE, A. W. C. (1957). Iodophors as disinfectants. *J. Amer. pharm. Ass.* (*Sci. Edn*) **46**, 500.

LAZARUS, N. E. (1952). New development in detergent-sanitizers. *Milk Dlr* **42**, 45.

LAZARUS, N. E. (1954). Iodine bactericides in the dairy industry. *J. Milk Tech.* **17**, 144.

MCCULLOCH, E. C. (1945). *Disinfection and Sterilization.* Philadelphia: Lea & Febiger.

MOORE, C. D. & HARDWICK, R. B. (1956). Germicides based on surface active agents. *Mfg Chem.* **17**, 305.

MUELLER, W. S. (1955). Bactericidal effectiveness of iodophor-detergent-sanitizers. *J. Milk Tech.* **18**, 144.

NEWBOULD, F. H. S. & BARNUM, D. A. (1956). Studies in sanitation in micrococcal mastitis. I. Teat dipping. *Canad. J. comp. Med.* **20**, 130.

NEWBOULD, F. H. S. & BARNUM, D. A. (1958). The effect of dipping cows' teats in a germicide after milking on the number of micrococci on the teat-cup liners. *J. Milk Tech.* **21**, 306, 348.

NEWBOULD, F. H. S. & BARNUM, D. A. (1960). The reduction of the microflora of milking machine inflations by teat dipping and teat cup pasteurization. *J. Milk Tech.* **23**, 374.

ORTENZIO, L. F. (1955). Report on fungicides and subculture media. *J. Ass. off. agric. Chem.* **38**, 274.

ORTENZIO, L. F., STUART, L. S. & FRIEDL, J. L. (1953). The resistance of bacterial spores to constant boiling hydrochloric acid. *J. Ass. off. agric. Chem.* **36**, 480.

PELTOLA, E. & MÄLKKI, Y. (1959*a*). Testing of detergent-sterilizer Iodosept. *Valt. Maitotalousk. Kone ja Tarvikekoetuksia*, 45.

PELTOLA, E. & MÄLKKI, Y. (1959*b*). Testing a detergent-sterilizer Pb. 1891. *Vali. Maitotalousk. Kone ja Tarvikekoetuksia*, 46.

ROHM & HAAS Co. (1954). Improvements in or relating to iodine complexes. Brit. Pat. no. 750,747.

(*Received* 21 *February*, 1962)

Fahmy, T. K., Hopton, J. W. & Woodbine, M. (1962). *J. appl. Bact.* **25** (2), 202–212.

THE MINERAL NUTRITION OF *HANSENULA* SPP. OF YEAST IN RELATION TO FAT PRODUCTION

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SUMMARY: The development of cultures of 'high fat' and 'low fat' species of *Hansenula* under different nutritional regimes in shaken culture has been examined. The cultures were sampled at frequent intervals throughout growth, and determinations made of cell number and cell mass, uptake of nitrogen and glucose and synthesis of fat and polysaccharide.

In all media there was an initial phase of rapid multiplication associated with nitrogen uptake, but appreciable increase in yeast mass occurred after the glucose had been exhausted and most of the accretion of fat and polysaccharide occurred during this phase of growth. In a nitrogen deficient medium the production of fat and polysaccharide was accentuated and, whereas the cell count of 'low fat' species increased during the second assimilatory phase, that of 'high fat' species did not. The behaviour of yeasts in a phosphorus deficient medium simulated that in a nitrogen deficient medium, but behaviour in a medium containing a low concentration of sulphur differed little from that in a complete medium.

The possibility of using micro-organisms for the conversion of carbohydrate to fat on an industrial scale has attracted the attention of numerous investigators and there is now an extensive literature on microbial fat production (see Woodbine, 1959). A number of yeasts and fungi have been shown to be capable of synthesizing large quantities of fat and it is well established that fat content is increased if growth takes place in an aerated medium of high carbohydrate to nitrogen ratio (i.e. relative nitrogen deficiency). However, the literature on microbial lipogenesis is notable for the conflict of opinion over the effect of the concentration and composition of the medium constituents on fat production. Much of the discord is attributable to the limited values of inferences drawn from experiments in which organisms are grown for an arbitrarily defined time and are then harvested and analyzed. Often the final analysis is related only to the starting conditions and no account is taken of differences in the progress of cultures growing under different regimes. Work such as that of Tornqvist & Lundin (1951) in which the cell mass was analyzed at intervals during the growth of a culture of *Rhodotorula gracilis* clearly showed that the change in composition can be large and indicated that the balance of synthetic activities of a cell at a particular time will be determined by the status of the environment at that time.

In a previous investigation (Hopton & Woodbine, 1960) it was shown that whereas some species of *Hansenula* (e.g. *H. saturnus* and *H. ciferrii*) produced relatively large amounts of fat when grown in a typical 'fat' medium, others (e.g. *H. subpelliculosa* and *H. angusta*) produced much smaller amounts. A more detailed examination of the effects of variation of the nitrogen and glucose concentrations, principally, of the medium, and of the phosphate and sulphate concentrations, on the development of cultures of these species (particularly of *H. saturnus*) has been undertaken and the results are presented in the present paper.

MATERIALS AND METHODS

General

Yeast species. The *Hansenula* yeasts used originated from the collection at the Northern Regional Research Laboratory, Peoria, Illinois, U.S.A. and have been fully described by Wickerham (1951). The species and collection designations were: *H. saturnus* Y-1304, *H. ciferrii* Y-1031, *H. subpelliculosa* Y-1683 and *H. angusta* Y-1798.

Media. In all experiments except one, completely synthetic media were used which contained (/litre) the following trace elements and vitamins: $CuSO_4.5H_2O$, 0·01 mg; $ZnSO_4.7H_2O$, 0·2 mg; $FeCl_3.6H_2O$. 5 mg; *meso*-inositol, 50 mg; nicotinic acid, 5 mg; pyridoxin, 1 mg; aneurin hydrochloride, 200 μg; riboflavin, 100 μg; *p*-aminobenzoic acid, 300 μg; Ca-D-pantothenate, 500 μg; D-biotin, 6 μg. In one experiment yeast extract at a concentration of 1·5 g/l was used in place of the trace elements and vitamin constituents. All media also contained (/litre): NaCl, 0·5 g and $CaCl_2.6H_2O$, 0·5 g, and variable concentrations of KH_2PO_4, $MgSO_4.7H_2O$, glucose and urea (the nitrogen source).

The media were constituted by combining in appropriate proportions sterilized concentrated solutions of minerals (yeast extract when used), vitamins (Seitz filtered), glucose and urea.

The volume of culture medium and the size of the culture flask varied and are stated in the text.

Inoculation. The inoculum culture was grown for 72 hr in a medium of composition (/litre): KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 1 g; NaCl, 0·5 g; $CaCl_2.6H_2O$, 0·5 g; $FeCl_3.6H_2O$, 0·005 g; yeast extract, 1·5 g; asparagine, 5 g; and glucose, 20 g. The yeast was recovered by centrifuging, washed once with distilled water, recentrifuged and suspended in distilled water to give a dry weight concentration of 4 mg/ml. The inoculum was a volume of this suspension equal to 1% of the volume of the medium.

Incubation conditions. The cultures were grown in flasks agitated on a rotary shaker at $26{\cdot}5 \pm 1{\cdot}0°$.

Analytical methods

All samples (10 or 20 ml) were taken from a single culture. They were centrifuged in tared centrifuge tubes, and samples of the supernatants were frozen and kept for pH, glucose and nitrogen determinations. The yeast mass was washed once with distilled water, centrifuged again and then brought to the original sample volume with distilled water. Samples of this suspension (usually 1 ml) were removed for the determination of polysaccharide and cell count. The remainder was centrifuged and the yeast pad was dried overnight at 80° and then weighed. The dried yeast was retained for fat estimation.

Cell number. This was determined using a haemocytometer slide. Cells with buds were counted as single cells.

Glucose estimation. The ferricyanide method of Hulme & Narain (1931) was employed.

Nitrogen. A conventional semimicro Kjeldahl method, with $CuSO_4$ as catalyst, was used. Ammonia was determined by the method of Yuen & Pollard (1953).

Yeast polysaccharide. This was determined by the anthrone method of Trevelyan & Harrison (1952). Glucose was used as the standard.

Yeast fat. Fat in the dried yeast was determined as described by Hopton & Woodbine (1960).

RESULTS

The relationship between cell numbers and cell mass in growing cultures. The four yeasts were grown in a medium with yeast extract as the trace element and vitamin source, the concentrations of KH_2PO_4, $MgSO_4.7H_2O$, urea and glucose being 1 g, 1 g, 0·46 g and 40 g/l respectively. The total nitrogen concentration, which included that in the yeast extract, was 330 mg/l. The medium was distributed in 200 ml amounts in 2 litre conical flasks. Samples of 10 ml of culture were removed at intervals of 12 hr for determinations of cell numbers and cell mass, and the results are shown in Table 1.

Table 1. *The relationship between cell numbers* and cell mass† in four species of* Hansenula

Incubation time (hr)	*H. saturnus*		*H. ciferrii*		*H. subpelliculosa*		*H. angusta*	
	Yield†	Count*	Yield	Count	Yield	Count	Yield	Count
0	4	2	4	2	4	3	4	2
12	107	71	224	170	211	201	85	136
24	673	219	575	197	648	214	376	176
36	946	221	764	‡	994	453	541	236
48	1241	‡	1054	233	1370	532	660	261
60	1346	238	1325	241	1688	665	776	‡
72	1428	269	1508	259	1753	762	1003	341
84	1360	‡	1528	288	1686	‡	1089	435
96	‡	‡	1429	‡	‡	‡	1182	567

* Cell numbers, in 10^6/ml. † Cell mass, as mg/100 ml. ‡ Estimation not done.

It is seen that multiplication of the low fat species *H. subpelliculosa* and *H. angusta* occurred throughout the incubation period, whereas multiplication of the high fat species *H. saturnus* and *H. ciferrii* practically ceased at a time when the cell mass was less than one half of the maximum eventually attained. In a further experiment *H. subpelliculosa* and *H. saturnus* were grown in a synthetic medium. The concentrations of KH_2PO_4, $MgSO_4.7H_2O$ and glucose were the same as in the previous experiment, but urea was included at a concentration equivalent to 302 mg of nitrogen/l. The pH of the medium as prepared was 4·7 and no adjustment was made. Flasks of 1,500 ml capacity containing 200 ml of medium were used and 10 ml samples were taken at frequent intervals during the incubation period.

The results of the cell count and yield determinations confirmed that the behaviour of the cultures was much the same as in the previous experiment, but the more frequent sampling revealed that at about the time of glucose exhaustion there was a phase of constancy in the cell count of the *H. subpelliculosa* culture. Determinations of nitrogen uptake, glucose consumption and yeast composition showed that the nitrogen was completely assimilated in a relatively short time and that the contrasts in fat and polysaccharide synthesis between the two species were particularly obvious during the phase when growth must have been taking place at the expense of products of glucose breakdown. With both yeasts the pH fell during the phase of glucose consumption and subsequently rose but little.

Table 2. *The constituent concentrations* and initial pH of the various media used*

Constituent	Type of medium			
	Low N	High N	Low SO_4	Low PO_4
Nitrogen (as urea)	30·2	150·8	150·8	150·8
KH_2PO_4	100	100	100	20
$MgSO_4.7H_2O$	100	100	20	100
$MgCl_2$	†	†	30·9	†
KCl	†	†	†	43·7
Initial pH	4·7	5·2	4·8	5·5

* Concentrations are in mg/100 ml. † None added.

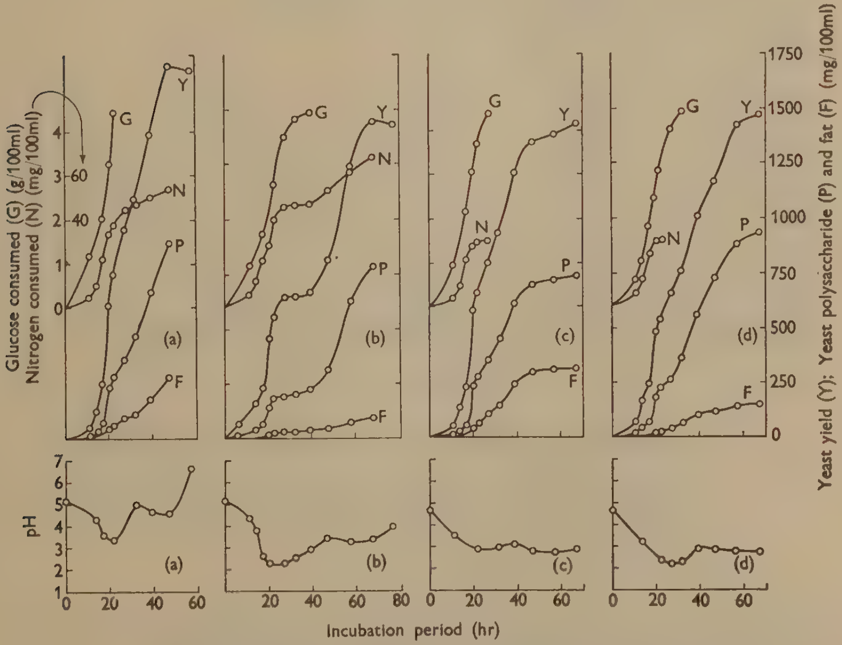

Fig. 1. The changes in yeast yield (Y), polysaccharide content (P), fat content (F), glucose consumption (G) and nitrogen consumption (N), all expressed in terms of culture volume, and in pH, in relation to time of incubation, in cultures of *H. saturnus* (a, c) and *H. subpelliculosa* (b, d) in media with high (a, b) and low (c, d) initial levels of nitrogen.

Effect of variation in nitrogen, phosphate and sulphate concentration. Fat synthesis is known to be enhanced under conditions of deficiency other than that of nitrogen and in a further experiment the growth of *H. saturnus* and *H. subpelliculosa* in media of low phosphate, low sulphate, low nitrogen and high nitrogen level were compared.

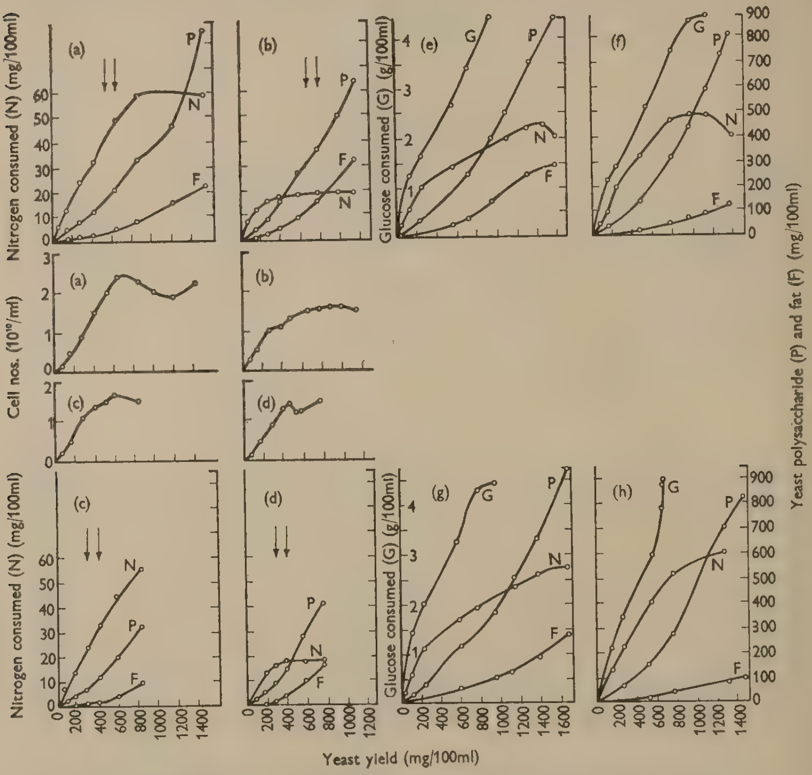

Fig. 2. The changes in polysaccharide content (P), fat content (F), glucose consumption (G) and nitrogen consumption (N), all expressed in terms of culture volume, and in cell numbers, in relation to yeast yield, in cultures of *H. saturnus* (a, c, e, g) and *H. subpelliculosa* (b, d, f, h) in media with high (a, b) and low (c, d) initial nitrogen levels and with low initial phosphate (e, f) and sulphate (g, h) levels.
The arrows indicate the approximate positions of complete glucose utilization.

Synthetic media were used and the concentrations of the constituents which were varied are shown in Table 2. The concentrations of Mg^{++} and K^+ were equalized by adding $MgCl_2$ or KCl as appropriate. Flasks of 1,500 ml capacity containing 200 ml of culture medium were used and 10 ml of culture were withdrawn at each sampling. Fig. 1 shows the results of the analyses for the high and low nitrogen cultures of both yeasts, expressed in relation to the age of the cultures, and in Fig. 2 the results for all the cultures are expressed in relation to yeast yield. With both yeasts the behaviour in the low sulphate medium resembled that in the high nitrogen medium, whereas the behaviour in the low phosphate medium was similar to that in the low nitrogen medium in that the fat and polysaccharide content of the yeast crop at maximum yield was greater than that of an equal weight of yeast crop in the high nitrogen medium. Moreover, the *H. subpelliculosa* culture showed no intermediate stationary phase. In all media both yeasts multiplied rapidly at first, but multiplication slackened when less than one half of the glucose had been consumed and subsequently increase in cell numbers was not commensurate with increase in yeast mass. Although the differences in the proportions of fat and polysaccharide formed by the two yeasts became evident during the phase of glucose breakdown, the contrast was sharper during the phase of growth on the products of glucose breakdown. However, there are other clear differences in the behaviour of the two yeasts. *H. subpelliculosa* growing in high nitrogen media exhibited an intermediate stationary phase which is reminiscent of the phenomenon of diauxie (the low phosphate medium is an interesting exception), and in the second phase nitrogen

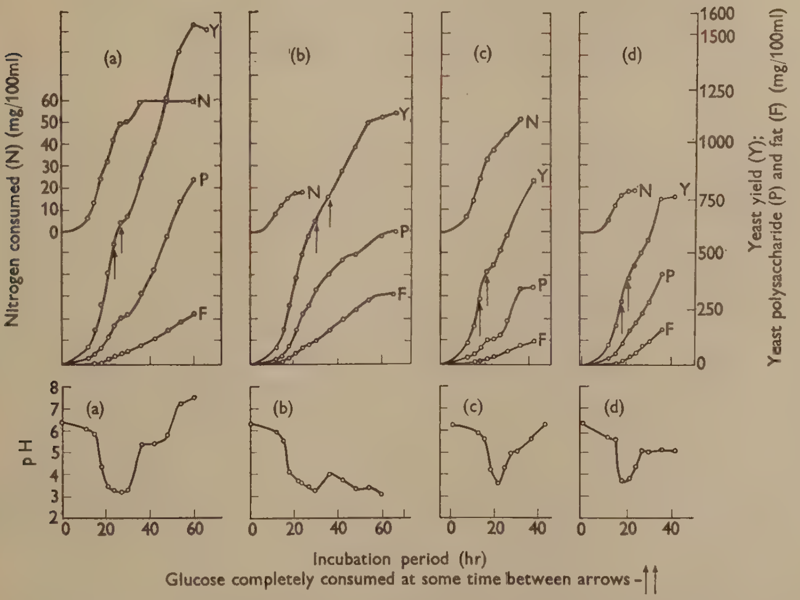

Fig. 3. The effect of different combinations of high and low initial levels of nitrogen (N) and glucose (G) on the growth and composition of *H. saturnus*, in relation to time of incubation.
Conditions: (a), high N, high G; (b), low N, high G; (c), high N, low G; (d), low N, low G.
The arrows indicate the approximate positions of complete glucose utilization.

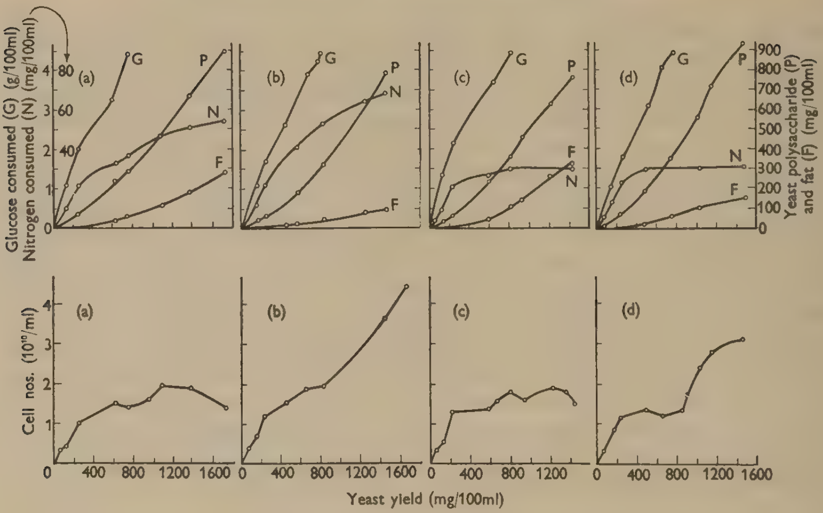

Fig. 4. The effect of different combinations of high and low initial levels of nitrogen (N) and glucose (G) on the growth and composition of *H. saturnus*, in relation to the amount of yeast produced.
Conditions: (a), high N, high G; (b), low N, high G; (c), high N, low G; (d), low N, low G.

was taken up and multiplication occurred. With *H. saturnus* there was no evidence of diauxie in high nitrogen media, and catabolism during the phase of glucose breakdown appeared to create an environment which curtailed nitrogen uptake and cellular multiplication during the second assimilatory phase.

Effect of variation of glucose and nitrogen concentration on the growth and composition of H. saturnus. All the foregoing results demonstrated that there was a marked change in the net synthesis of fat and polysaccharide when glucose ceased to be available as substrate. In a further experiment the effect on the pattern of development of cultures of *H. saturnus* of varying the glucose and nitrogen concentrations of the medium was investigated. The glucose and nitrogen (as urea) concentrations, respectively, of the four media were 4 g and 74·5 mg, 4 g and 18·5 mg, 2 g and 74·5 mg and 2 g and 18·5 mg/100 ml. To accentuate the effect of nitrogen deprivation the lower level of nitrogen concentration was less than that of any medium previously used and the initial pH of all the media was raised to 6·2–6·3 to enhance the buffering capacity. The media, which were synthetic and had KH_2PO_4 and $MgSO_4.7H_2O$ both included at a concentration of 1 g /l, were distributed in 320 ml amounts in 1,500 ml conical flasks and samples of 20 ml of culture were taken at 3 or 6-hourly intervals, beginning at the 12th hour after inoculation. The results of the experiment are shown in Figs. 3 and 4. The progress of cell mass increase in all the cultures was much the same as had been observed previously with *H. saturnus*, with a clear change in rate after the glucose had been exhausted. However, the cultures in the media with a higher nitrogen level showed a short stationary phase at the termination of glucose uptake, similar to that exhibited by cultures of *H. subpelliculosa* but much shorter. The higher initial pH had a beneficial effect on cell multiplication, which was sustained for longer than had hitherto been observed; curtailment of active multiplication appeared to be associated with glucose exhaustion rather than nitrogen limitation, except in the culture containing the lower level of nitrogen and the higher level of glucose, in which exhaustion of nitrogen apparently contributed to the fall off in cell multiplication. A comprehensive examination of the state of the cells during the growth of the low nitrogen, high glucose culture represented by Figs. 3 and 4b showed that budding had almost ceased at the 24th hour (Table 3). The pH changes were complex and of a pattern similar to that observed in earlier experiments. They suggest sequential utilization of available substrates, but the pronounced rise in pH late in the high nitrogen cultures was probably caused by the action of urease on nonutilized urea. As in previous experiments the phase of glucose breakdown was a phase of increasing acidity, but in these cultures the fall was through a higher

Table 3. *The changes in count of* H. saturnus *with time in a low nitrogen, high glucose medium and the proportions of single cells and cells with one and two buds*

Incubation time (hr)	Cell count (10^8/ml)	Total no. of cells counted	No. of single cells	No. of cells with one bud	No. of cells with two buds
0	3	533	525	8	0
12	19	757	474	238	45
15	51	1,017	575	442	0
18	101	1,034	737	297	0
21	105	843	755	88	0
24	134	534	528	6	0
27	145	579	579	0	0
30	139	555	555	0	0
36	144	576	576	0	0
42	167	334	334	0	0
48	154	308	308	0	0
54	144	288	288	0	0
60	152	303	303	0	0

Table 4. *The polysaccharide and fat contents and yields of* H. saturnus *grown in media containing high and low concentrations of nitrogen, glucose and phosphate*

Concns of nitrogen (N), glucose (G) and phosphate (P)	Maximum yield (mg/100 ml)	Polysaccharide (%)	Fat (%)
High N, G and P	1,542	54·0	12·8
High N and G; low P	1,549	47·4	13·8
High N and P; low G	836	38·1	11·9
Low G and P; high N	835	38·3	11·7
High G and P; low N	1,144	52·0	27·6
Low N and P; high G	1,022	49·4	28·4
Low N and G; high P	778	57·4	23·2
Low N, G and P	754	50·2	20·0

pH range than hitherto and this may explain the longer near-proportional uptake of nitrogen by the increasing cell mass. These results also confirm that in media of low nitrogen concentration fat and polysaccharide synthesis become dominant during the first phase of glucose breakdown. In media of higher nitrogen concentration protein synthesis apparently predominates until the glucose is exhausted, and in the medium with the lower concentration of glucose it continues throughout growth.

In a further experiment the conditions of growth were similar in all respects, but the KH_2PO_4 concentration of the media was reduced to 0·5 g/l. This is 2·5 times as high as the previous low concentration of phosphate (Table 2); it had only a marginal effect on cell composition, but caused a reduction in yield in the nitrogen deficient cultures (Table 4).

DISCUSSION

When *Saccharomyces cerevisiae* grows in an aerated glucose medium the first phase is essentially fermentative and the cell yield is low. As the glucose concentration falls aerobic catabolism increases and cell yield also increases. In the second phase of growth, which may be preceded by a diauxic phase, ethanol produced in the first phase is utilized (Lemoigne, Aubert & Millet, 1954; Slonimski, 1956). Growth at the expense of glucose is unusual in that it is characterized by a high rate of catabolism with a low efficiency of cell production (Magasanik, Neidhardt & Levin, 1958).

In this investigation the *Hansenula* spp. have been found to behave in a similar manner. Irrespective of the nutritional status of the medium there is at first a rapid multiplication, the length of which is dependent on a lowering of the pH, associated with extensive glucose dissimilation and low yield of organisms. If the growth in media containing a plentiful supply of nitrogen is first considered it is seen that during this first phase the bulk of the yeast consists of nitrogenous material. Most of the remainder is polysaccharide and little fat is synthesized. *Hansenula* yeasts are known to produce ethanol, acetic acid and ethyl acetate from glucose (Davies *et al.* 1951) and the proportions in which these compounds are found depends on the species and the pH of the medium (Davies *et al.* 1951; Peel, 1951; Tabachnick & Joslyn, 1953). Hence, at the termination of glucose dissimilation the yeast mass will be in an environment, the physical and chemical characteristics of which will have been determined by the properties of the species and the prior condition of the environment, e.g. initial pH, buffering capacity and glucose concentration. Subsequent development of the culture will be determined by the manner in which the yeast mass is able to handle the dissimilation products of glucose catabolism and indeed it is during this second phase of growth that the 'high fat' species *H. saturnus* and the 'low fat' species *H. subpelliculosa* show their most marked differences in behaviour. However, during the first phase both species may have produced intermediates which are eminently suitable for fat and polysaccharide synthesis and it is perhaps not surprising, therefore, that synthesis of these cell constituents predominates in the second phase. With *H. saturnus* there is no further appreciable cell multiplication and although there is a phase in which pH increases and some nitrogen uptake occurs (this is shown clearly in the culture represented by Fig. 3c), the bulk of the cell mass increase is accounted for by polysaccharide and fat. Fat accounts for about 20% of this accretion. *H. subpelliculosa* exhibits a definite diauxic phenomenon and both cell multiplication and nitrogen uptake occur in the second phase; but polysaccharide is the major product of cellular synthesis. The behaviour of *H. saturnus* in the second phase may be attributable to exhaustion of an essential vitamin or to inhibition of normal growth by metabolic products; however, it is tempting to speculate that this species can proceed with assimilation by means of preformed enzymic equipment but is unable to adapt itself to augment its nitrogen metabolism. *H. subpelliculosa* on the other hand exhibits a typical glucose inhibition (Neidhardt, 1960) and may be able in the second phase to elaborate enzymes which augment nitrogen supply. Barnett & Kornberg (1960) have presented evidence for the operation of the glyoxylate cycle in yeasts growing on acetate. Interestingly enough, deprivation of phosphate abolishes the glucose effect with *H. subpelliculosa* and Englesberg (1959) has postulated that interference with phosphate metabolism may be responsible for the abolition of glucose inhibition in *Salmonella typhi-murium*.

If growth in conditions of relative nitrogen deficiency is now considered, it is seen that even during the first phase of growth polysaccharide and fat synthesis are more extensive than in cultures growing in nitrogen-sufficient conditions. During metabolism the intermediates from glucose dissimilation will be distributed to three main cellular systems—those dealing with protein, polysaccharide, and fat synthesis. The results suggest that when protein synthesis becomes restricted because of nitrogen deprivation the intermediates are channelled predominantly through the polysaccharide and fat pathways and that this continues as long as cellular synthesis can occur. *H. saturnus* and *H. subpelliculosa* differ particularly in the second phase of growth, in that *H. saturnus* appears to divert more intermediates through the fat system at the expense of the polysaccharide system than does *H. subpelliculosa* (cf. Fig. 1, c and d). A diversion of this kind may explain the behaviour of *H. anomala* reported by Brock (1956). With *H. subpelliculosa*, although the glucose effect is abolished—and this appears to contradict the prediction that nitrogen deficiency should accentuate inhibitory effects (Neidhardt, 1960)—multiplication can occur and this may indicate extensive enzyme reorganization in spite of, or indeed perhaps because of, lack of exogenous nitrogen.

In this investigation the authors have been interested primarily in the manner in which cell constitution changes in response to alteration in the environment. However, it is clear that before really worthwhile conclusions can be drawn more information about the nature, concentrations and fate of intermediate products is required. Moreover, greater control of environmental change is desirable and, since this work was completed, Herbert (1961) has made a plea that greater attention should be paid to environment control and has shown the advantages to be gained from this approach.

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REFERENCES

BARNETT, J. A. & KORNBERG, H. L. (1960). The utilization by yeasts of acids of the tricarboxylic acid cycle. *J. gen. Microbiol.* **23**, 65.

BROCK, T. D. (1956). Lipid synthesis in *Hansenula anomala*. *Mycologia* **48**, 337.

DAVIES, R., FALKINER, E. A., WILKINSON, J. F. & PEEL, J. L. (1951). Ester formation by yeasts. I. Ethyl acetate formation by *Hansenula* species. *Biochem. J.* **49**, 58.

ENGLESBERG, E. (1959). Glucose inhibition and the diauxie phenomenon. *Proc. nat. Acad. Sci., Wash.*, **45**, 1494.

HERBERT, D. (1961). The chemical composition of micro-organisms as a function of their environment. In *Microbial Reaction to Environment*, p. 391. Symp. Soc. Gen. Microbiol. no. 11. Cambridge: University Press.

HOPTON, J. W. & WOODBINE, M. (1960). Fat synthesis by yeasts. I. A comparative assessment of *Hansenula* species. *J. appl. Bact.* **23**, 283.

HULME, A. C. & NARAIN, R. (1931). The ferricyanide method for the determination of reducing sugars. A modification of the Hagedorn-Jensen-Hanes technique. *Biochem. J.* **25**, 1051.

LEMOIGNE, M., AUBERT, J. P. & MILLET, J. (1954). La production d'alcool et le rendement de croissance de la levure de boulangerie cultivée en aérobiose. *Ann. Inst. Pasteur* **87**, 427.

MAGASANIK, B., NEIDHARDT, F. C. & LEVIN, A. P. (1958). *Physiological Adaptation*, p. 159. Ed. PROSSER, C. L. Washington, D.C.: American Physiological Society.

NEIDHARDT, F. C. (1960). Mutant of *Aerobacter aerogenes* lacking glucose repression. *J. Bact.* **80**, 536.

PEEL, J. L. (1951). Ester formation by yeasts. 2. Formation of ethyl acetate by washed suspensions of *Hansenula anomala*. *Biochem. J.* **49**, 62.

SLONIMSKI, P. P. (1956). Respiration and the growth of yeast. *Monogr. Soc. chem. Ind. Lond.* no. 3, p. 7.

TABACHNICK, J. & JOSLYN, M. A. (1953). Formation of esters by yeast. II. Investigations with cellular suspensions of *Hansenula anomala*. *Plant Physiol.* **28**, 681.

TORNQVIST, E. & LUNDIN, H. (1951). The biotechnical production of fat. Using cane molasses as raw material. *Int. Sug. J.* **53**, 123.

TREVELYAN, W. E. & HARRISON, J. S. (1952). Studies on yeast metabolism. 1. Fractionation and microdetermination of cell carbohydrates. *Biochem. J.* **50**, 298.

WICKERHAM, L. J. (1951). Taxonomy of yeasts. *Tech. Bull. U.S. Dep. Agric.* no. 1029.

WOODBINE, M. (1959). Microbial fat: micro-organisms as potential fat producers. In *Progress in Industrial Microbiology* **1**, 179.

YUEN, S. H. & POLLARD, A. G. (1953). Determination of nitrogen in soil and plant materials: use of boric acid in the micro-Kjeldahl method. *J. Sci. Fd Agric.* **4**, 490.

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McCoy, J. H. (1962). *J. appl. Bact.* **25** (2), 213–224.

THE ISOLATION OF SALMONELLAE

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1. Introduction

The isolation and identification of salmonellae no longer falls almost exclusively within the province of medical and veterinary bacteriologists. In the past decade, the presence of salmonellae, often in large numbers and sometimes with a wider range of serotypes than encountered in human or animal infections, has been demonstrated in native (Murdock, 1954; Report, 1955; Report, 1959*a*) and imported egg products (Report, 1958); in native and imported animal feeding stuffs and fertilizers (Walker, 1957; Report, 1959*b*; Report, 1961); in native (McDonagh & Smith, 1958) and imported carcase meats (Hobbs & Wilson, 1959); in imported desiccated coconut (Galbraith, Hobbs, Smith & Tomlinson, 1960; Anderson, 1960); and in the environment and products of the broiler industry (Dixon & Pooley, 1961). Any one of these products may be used in apparently unassociated industries, as with imported dried bones which are used in the manufacture of gelatin for human consumption; in the fertilizer industry as plant food; and in animal feeding stuffs as a source of essential minerals. Several contaminated products may be used in one industry. For example, egg products and desiccated coconut in the confectionery trade, and egg products, carcase meats and gelatin in the manufactured meat products industry.

In some imported materials the numbers of salmonellae present are small; for example, in desiccated coconut (Galbraith *et al.* 1960). This, however, in no way lessens the significance of their presence, because in the normal processes of manufacture into human food these few organisms may find an environment suitable for multiplication (Thomson, 1953).

Although few of the materials mentioned above are free from one or other of the normal inhabitants of the animal intestine, in many materials the association of these organisms with salmonellae is erratic and unpredictable (Hobbs & Wilson, 1959; Galbraith *et al.* 1960), so that the potential presence of salmonellae in the material cannot be inferred indirectly as in the examination of water and shellfish; it must be demonstrated by direct examination. The small numbers of salmonellae present in some foodstuffs or in their raw materials does, however, mean that their presence is unlikely to be demonstrated by direct plating on selective media, and that enrichment followed by plating on a selective medium or media is essential.

2. FACTORS INFLUENCING THE ISOLATION OF SALMONELLAE

(a) *Size of inoculum*

Within limits, the larger the quantity of sample examined the higher is the percentage of positive samples. Table 1 shows the incidence of salmonella isolations from increasing volumes of a sewage polluted water. A sixfold increase in the volume of sample examined resulted in more than doubling the percentage of salmonella isolations. In the same way, salmonellae were not isolated from a fish meal until the weight of sample examined had been increased to a minimum of 100 g. For crude sewage, and sewage effluent, 100 ml volumes of samples are recommended. For sewage polluted natural waters where the sewage has undergone considerable dilution, 2,500 ml quantities are recommended.

Table 1. *The relationship between size of inoculum and incidence of salmonella isolations*

Nature and size of sample		No. of samples		Positive samples (%)
		Tested	Positive	
Sewage polluted water	450 ml	200	66	33·0
	1,350 ml	88	44	50·0
	2,700 ml	35	25	71·4
Fish meal	40 g	19	0	0·0
	100–150 g	13	3	23·1

Volumes of fluids up to 200 ml may conveniently be added to an equal volume of enrichment medium in a 1 lb Kilner jar. Greater volumes are handled more conveniently by filtering the liquid through a plug of sterile absorbent cotton wool inserted in the neck of a sterile funnel. After filtration, the plug is immersed in the enrichment medium and incubated. For foods, 50 g of sample may be conveniently examined either in one lot or in two lots of 25 g.

With heavily contaminated materials (e.g. the fluid expressed from sewer swabs, and samples of bone meals) a greater yield of salmonellae is obtained by diluting the material before enrichment (Lendon & Mackenzie, 1951). The fluid expressed from the swab should be diluted 1:10, 1:100 and 1:1,000 and equal volumes of the dilutions inoculated into enrichment broths. Fluid samples may be diluted with normal saline or quarter-strength Ringer's solution, and particulate samples, e.g. bone meals, with sterile sand.

A ratio of inoculum to enrichment medium of 1:4 to 1:6 has in general been found most satisfactory. As a standard procedure 25 g of sample are added to 150 ml of enrichment medium in a 1 lb Kilner jar.

(b) *Choice of enrichment medium*

For many materials and foodstuffs the use of an enrichment medium is unnecessary, as a solution or suspension of the material in water, normal saline or quarter-strength Ringer's solution forms a good medium for bacterial growth. This method was found to be of value for the isolation of salmonellae from spray dried egg (Report, 1947). More recently it has been used successfully for the isolation of salmonellae from a variety of contaminated materials, including bone meals and bone flours, blood meal, meat meals, meat and bone meals, dried sewage sludge, and egg products including dried and crystalline egg and liquid and frozen whole egg. Incubation of such solutions at the selective temperature of 43° (Harvey & Thomson, 1953) facilitates the isolation of salmonellae.

Incubation in water, normal saline or quarter-strength Ringer's solution is not suitable for the isolation of salmonellae from desiccated coconut, gelatin, and liquid or crystalline egg albumen. For many materials heavily contaminated with other organisms, such as crude sewage and sewage effluent, bone meals, meat and bone meals, liquid and frozen egg, and made-up meat products, the use of a selective or enrichment medium is desirable.

The enrichment media recommended are selenite F medium (Hobbs & Allison, 1945; Smith, 1959) and Rolfe's (1946) modification of tetrathionate broth. Generally, small volumes or quantities of samples are inoculated into single strength enrichment broths, but where the volume of inoculum equals that of the enrichment medium the use of double strength enrichment medium is common.

Various methods of increasing the yield or ease of isolation of salmonellae from enrichment broths have been proposed. Harvey & Thomson (1953) recommended the use of a selective temperature, 43°, for the incubation of selenite enrichments. Jameson (1961) found that 'secondary' enrichment of negative broths in either the primary medium or a different medium produced a high yield of salmonellae.

A series of comparisons of two enrichment media, involving strength of medium, selective temperature and three varieties of secondary enrichment, was carried out. Tetrathionate broth incubated at 43° was not included, because a preliminary series of tests showed that at this temperature tetrathionate is lethal to salmonellae and to most organisms. An opportunity has not yet occurred to compare the Rolfe (1946) B and Jameson (1961) modifications of this medium.

The inoculum used with each medium in this series of comparisons was obtained by collecting hourly samples over 24 hours once weekly of a sewage effluent. Each series of comparisons occupied 5–6 weeks. A 25 ml portion of each hourly sample was inoculated into bottles containing 25 ml of the medium under test, incubated at 37° and subcultured daily for 4 days.

Table 2. *The results of a series of comparisons of two enrichment media* for the isolation of salmonellae from samples of a sewage effluent*

Comparison medium 2† with details of procedure	No. of samples tested	Positive samples (nos.)			Positive samples (%)		
		Total	Med. 1*	Med. 2	Med. 1*	Med. 2	Diffce‡
Tetrathionate, SS, 37°	111	49	33	35	67·3	71·4	−4·1**
Tetrathionate, DS, 37°	70	10	6	6	60·0	60·0	0·0**
Selenite, SS, 37°	122	54	29	36	53·7	66·7	−13·0**
Selenite, SS, 43°	110	31	26	13	83·9	41·9	42·0§
Tetrathionate, SS, 37°; secondary enrichment, tetrathionate 24 hr	110	56	34	42	60·7	75·0	−14·3**
Tetrathionate, SS, 37°; secondary enrichment, tetrathionate 48 hr	107	5	3	2	60·0	40·0	20·0**
Tetrathionate, SS, 37°; secondary enrichment, selenite, 24 hr	83	6	4	3	66·7	50·0	16·7**

* Medium 1 was always tetrathionate broth B, single strength, incubated at 37°. † Medium 2: SS, single strength; DS, double strength. ‡ Difference: percentage of samples positive in medium 1 minus the value for medium 2. § This value is significant at the 5% level. ** Not significant.

A preliminary series of comparisons with the same medium was carried out to determine the experimental error involved, and in each comparison of two media the standard medium was single strength tetrathionate B broth at 37°. The results of this series are given in Table 2. No difference in efficiency was shown between single-strength and double-strength tetrathionate broth, or between tetrathionate broth at 37° and selenite broth at 37°. However, isolations from selenite broth incubated at 43° were significantly fewer. No advantage resulted from secondary enrichment either into the same medium after 24 or 48 hr incubation of the primary enrichment or into another medium, selenite, after 24 hr primary enrichment in tetrathionate. Although no difference was found in the yield of salmonellae from portions of the same sample enriched in selenite and tetrathionate B broths, it is recommended that both broths be used for the routine examination of samples. The broths are complementary. Selenite broth is superior to tetrathionate B broth for the isolation of *Salmonella typhi*, but tetrathionate B broth supports the growth of a wider range of serotypes than does selenite broth.

(c) *Optimum time for subculture from enrichment broths*

Many times and combinations of times for subculture from enrichment broths are recommended in the literature; 24 and 48 hr, and 24 and 72 hr are the combinations most frequently recommended.

To investigate the effect of time of subculture, cultures were made from a series of samples of sewage polluted water, of which a standard volume (450 ml) was enriched in tetrathionate broth at 37° and subcultured for 24, 48, 72 and 96 hr. The results are presented in Table 3. It is apparent that there was no one time at which all the positive samples were detected. Positives continued to appear, though in diminishing numbers, as long as subculture continued. Subculture for 24 and 48 hr would have selected rather more than 85% of the positive specimens, subculture at 24 and 72 hr rather less than 78%.

Table 3. *Details of the first appearance of salmonella positive subcultures in tests of samples of sewage polluted waters, and of their detection in subsequent subcultures*

Time of subculture (hr)	First positive subcultures		Nos. of first positives still positive on subsequent test at		
	No.	% of total	48 hr	72 hr	96 hr
24	60	63·2	34	15	11
48	21	22·1	—	10	4
72	4	4·2	—	—	1
96	10	10·5	—	—	—
Totals	95	100·0			

Table 4 relates the first time of appearance of salmonellae on subculture to the number of salmonellae in the inoculum, in a further series of sewage polluted waters (100 ml inoculum) enriched in tetrathionate at 37° and subcultured for 24, 48, 72 and 96 hr. In samples where the most probable number (MPN) of salmonellae in the inoculum was two or less, positives continued to appear for the first time at all times of subculture. In samples where the MPN ranged from 3·5 to 9, 26 out of 28 samples (approximately 93%) were positive after 48 hr incubation, and where the MPN exceeded 12 all samples were positive after 24 hr incubation.

Table 4. *The relationship between the time of appearance of the first salmonella positive subculture and the number of salmonellae in the inoculum*

No. of salmonellae in inoculum (MPN/100 ml)	No. of samples	Nos. of samples giving first positive subcultures after (hr)			
		24	48	72	96
1	52	16	18	10	8
2	22	9	8	4	1
3·5	10	7	3		
5	6	3	1	2	
7	7	5	2		
9	5	3	2		
12	3	3			
16	4	4			
23	1	1			
>23	3	3			

These findings suggest that the major factor determining the time of appearance of positive subcultures is the number of salmonellae in the inoculum. As this can never be known in advance the time of subculture and the number of subcultures necessary will be a matter of expediency. It is suggested that for the qualitative examination of samples subculture for 24 and 48 hr, which will select at least 80% of those containing salmonellae, should suffice. For quantitative examinations, daily subculture for 4 days would appear to be essential.

(d) *Choice of selective medium for subculture of enrichments*

The choice of a selective medium for subculture is limited by two facts. First, the existence of a lactose fermenting strain of salmonella, *Salm. ferlac*, which is approximately the 6th most common salmonella isolated from desiccated coconut; and second, the presence in faeces, crude sewage, sewage effluent, sewage polluted natural waters, and human and animal foods, of large numbers of nonpathogenic intestinal organisms which either fail to ferment lactose or ferment lactose late. As an extreme example of the difficulties attending the isolation of salmonellae from sewage, Houston (1914) examined 23,353 colonies of lactose nonfermenters arising from crude sewage by direct plating, without detecting a single colony of the typhoid bacillus. More recently Moore (1950) examined 250 out of 1,300 such colonies from deoxycholate platings of sewage known to contain *Salm. typhi* without detecting a single typhoid bacillus.

Although the exclusion of nonpathogenic lactose nonfermenters has been made easier by the introduction of screening media (Christensen, 1946; Gillies, 1956), nevertheless the task of identifying the colonies of pathogenic organisms among the profusion of colonies of lactose nonfermenters produced by subculture of heavily contaminated material on bile salt-lactose media is formidable. Harvey (1956) compared bile salt-lactose media with bismuth-sulphite agar and with brilliant green-MacConkey's agar and recommended the latter. The bismuth-sulphite medium (Wilson, 1938), however, possesses the advantage that on it salmonellae produce a characteristic colony which can be identified on sight (Plate 2, f). The original bismuth-sulphite medium of Wilson & Blair (1927) was devised for the isolation of *Salm. typhi* from sewage and sewage polluted waters and as such is rather inhibitory to other salmonellae, but the 1938 modification is suitable for the isolation of almost all serotypes of salmonellae. For the isolation of *Salm. typhi* the medium is used directly after pouring, but Cook (1952) found that freshly prepared plates were inhibitory to other serotypes. This inhibition lessened as the medium aged and small inocula of many serotypes grew well on media stored in the refrigerator for 4–5 days after preparation. This 'ageing' of the medium is essential for the production of the characteristic salmonella colony (see Plates 1 and 2). On properly aged bismuth-sulphite media characteristic salmonella colonies are easily recognized after 18 hr, but if freshly poured or insufficiently 'aged' plates are used 48 hr is usually necessary.

(e) *The characteristic salmonella colony*

According to Fry (1946, pers. comm.), on bismuth-sulphite agar (Wilson, 1938) suitably aged, salmonellae produce, after 18 hr, colonies characterized by a jet black circular centre with sharp edge, surrounded by a clear, translucent periphery. In cross section the colony is usually convex; but flat, umbilicate or circumvallate colonies are not unusual and all these varieties may be met with in a pure culture of a single serotype. The surface of the colony is invariably smooth (Plate 1, Plate 2, a and b). *Salm. typhi* can be recognized on sight as the colony is rather flatter, angular rather than circular, and the jet black centre presents a slightly matt appearance. The colony may be surrounded by a mirror of precipitated bismuth, but this mirror is common to all organisms which produce hydrogen sulphide and is not confined to salmonellae. The mirror is not a prominent feature of colonies on the 1938 modification of the medium, in which iron citrate replaces ferrous sulphate.

Colonies arising from a serotype in phase 1 are convex, resembling the eye of a rabbit. In these colonies the portion of the diameter taken up by the jet black centre greatly exceeds that occupied by the clear translucent periphery (Plate 2, a). Colonies arising from a serotype in phase 2 (Plate 2, b) are flat, and the portion of the diameter occupied by the clear translucent periphery greatly exceeds that occupied by the jet black centre. Generally, on primary isolation most serotypes are in phase 1, the majority of the colonies being of the rabbit's eye type. Very occasionally, a serotype in phase 2 produces an atypical colony in which the jet black centre is absent. The central portion of the colony, however, is coloured by the brilliant green in the medium and contrasts with the clear translucent periphery. Only three such strains have been met in the past 10 years, two of which were *Salm. typhi-murium* and the third *Salm. paratyphi* B.

Salm. gallinarum and *Salm. pullorum* also produce an atypical colony, green from dye absorption, with the centre more deeply stained. The colony is much smaller than that of other serotypes.

The colonies produced by *Salm. cholerae-suis* are invariably atypical, though most strains can be induced to produce typical colonies by repeated subculture. The colonies produced are clear and translucent, without either the jet black centre or the green centre resulting from the absorption of dye described above. The colony can be recognized with experience.

For development of the characteristic features good separation of the colonies is necessary, and Moore (1950) recommended plating equal volumes of three serial fivefold dilutions of the enrichment broth. In practice, inoculation of a whole plate with a wet loop has been found satisfactory. With crowded plates the use of a hand lens aids the recognition of characteristic colonies and even a single salmonella colony on such a plate can easily be identified.

3. The Identification of Salmonella Serotypes

Under a plate microscope, characteristic colonies are picked to slopes of Hartley's agar which are incubated in a water bath at 37°. After 4 hr incubation there is sufficient growth on the slopes for identification by direct slide agglutination, using polyvalent 'O', polyvalent 'H', specific and nonspecific salmonella sera, and *Salm. typhi* Vi serum (Memorandum, 1961). It should be remembered that whereas polyvalent salmonella 'O' serum contains antibodies sufficient to agglutinate with only some 200 out of the more than 700 recognized serotypes, polyvalent salmonella 'H' serum contains antibodies to all but a few of these serotypes. Characteristic salmonella colonies should therefore never be discarded because of failure to agglutinate with polyvalent 'O' serum or even because of some biochemical abnormality, but should be sent to the nearest Salmonella Reference Laboratory for identification. Characteristic salmonella colonies picked from the medium may be agglutinated directly without preliminary incubation when specimens of human or animal origin likely to contain only a single serotype are under examination. Foods, animal protein and minerals used in the manufacture of animal feeding stuffs, crude sewage, sewage effluent, and sewage polluted natural waters may contain salmonellae of many serotypes (Table 5), and direct agglutination of colonies picked in succession from the plate may lead to discordant results. In addition, it is convenient to have for slide agglutination more material than is contained in a single colony.

Table 5. *A list of materials tested for salmonellae, with the numbers of serotypes isolated therefrom*

Type of material and source		No. of samples positive	Nos. of samples with 1 and up to 6 serotypes						Samples with one serotype only	
			1	2	3	4	5	6	No.	%
Human foods	Meat products	23	22	1					22/23	95·7
	Egg products	110	109		1				109/110	99·1
	Coconut	45	44	1					44/45	97·8
Human infections. Human		1,099	1,092	6		1			1,092/1,099	99·4
Domestic sewage and effluent		90	87	2	1				87/90	96·7
Individual animals. Animals		78	76	1	1				76/78	97·4
Animal foods, complete		10	9	1					9/10	90·0
Sewer swabs. Pig abattoir		25	13	9	1	2			13/25	52·0
Raw materials	Marine products	28	14	6	1	1	3	3	14/28	50·0
	Meat products	30	11	10	7	2			11/30	36·7
	Bone products	4	1	2	1				1/4	25·0
Natural waters, receiving domestic crude sewage and industrial effluents		230	137	64	20	6	1		137/230	59·6

From plates, with the exception of those believed to contain only a single serotype, a number of colonies should be selected for examination. Twenty is a convenient number, though in material such as sewage receiving salmonellae from many sources, bone meals, meat and bone meals, and sun-dried fish meals, the number of serotypes isolated increases with the number of individual colonies picked for identification.

4. Salmonella Counts

The estimation of the most probable number of salmonellae/g of solid material or /100 ml or /litre of liquids is easily determined by the inoculation of measured quantities of sample into a number of tubes of enrichment medium. The tubes are incubated at 37° and subcultured for 4 days, the final number of positive tubes being recorded. The MPN of salmonellae present is then read off directly by reference to a table. A suitable table for 10 tubes each containing 0·1 ml or 0·1 g of the sample is given in Report (1959*c*). This table is capable of infinite expansion, as the MPN varies inversely with the sample volume, and it can therefore be used for samples ranging in size from 1,000 ml or g to 0·0001 ml or g. Quantitative examination of samples is essential for the assessment, for example, of the efficiency of heat treatment of liquid egg, or for the demonstration of seasonal changes in the salmonella content of sewage polluted waters. In this latter connection at least, qualitative examination alone does not disclose the seasonal variation (Fig. 1).

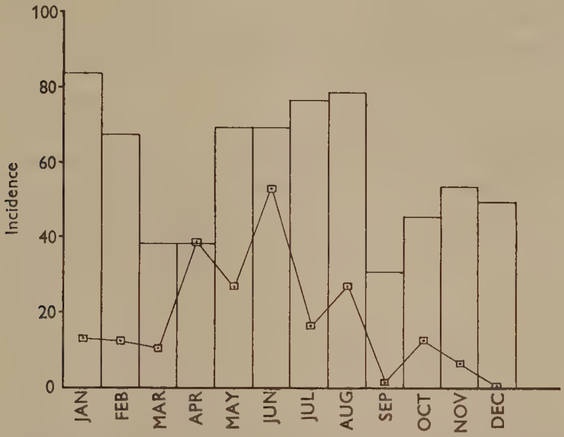

Fig. 1. The seasonal incidence of salmonellae in polluted water during 1961, expressed in terms of percentage of samples positive (unshaded areas) and MPN/1,000 ml (continuous line).

5. Acknowledgments

The table of most probable numbers reproduced in the Appendix appeared in Report (1959*c*) and is reproduced by kind permission of the *Journal of Hygiene*.

My thanks are due to Mr. J. Moore for technical assistance in the preparation of the many media required, and to Mr. G. E. Spain, F.I.M.L.T., for technical assistance in the identification of salmonellae; and to Mr. S. E. Veronique, A.I.B.P., A.R.P.S., for the photographs.

6. Appendix

(a) *Modified bismuth-sulphite agar*

The bismuth-sulphite medium used is based on 'Oxoid' (single pack) bismuth-sulphite agar, a modification of the medium of Wilson & Blair (1927). In the bismuth-sulphite medium of Wilson (1938) iron citrate is substituted for ferrous sulphate and the author pointed out that with one particular peptone black colonies failed to develop unless the sodium sulphite was reduced by half, the other constituents remaining the same.

Since the production of characteristic salmonella colonies rather than black colonies is desirable, the medium has been further modified. The medium in routine use is prepared as follows. The basic medium contains: Yeastrel, 4 g; peptone, 50 g; agar, 120 g; ferric citrate scales, 2 g; brilliant green, 1% (w/v), 5 ml; distilled water, 5,000 ml. The ingredients are dissolved by heating in the steamer, the solution is bottled in 500 ml amounts and autoclaved for 20 min at 10 lb/in^2. An indicator is also prepared, containing bismuth ammonio-citrate, 3 g; sodium sulphite (anhyd.), 10 g; *di*-sodium hydrogen orthophosphate (anhyd.), 5 g; dextrose, 5 g; distilled water, 100 ml. This mixture is brought to the boil, allowed to cool to room temperature and added to 1 litre of the basal medium at 55°.

(b) *Calculation of most probable numbers*

The following Table gives values for the MPN and the approximate 5% confidence limits, for a dilution test with 10 tubes each containing 0·1 ml of the sample.

No. of positive tubes	MPN (/100 ml)	Limits (in 39/40 samples)	
		Lower	Upper
0	—	—	369
1	105	15	749
2	223	56	895
3	357	114	1,110
4	511	190	1,380
5	693	283	1,690
6	916	400	2,100
7	1,200	548	2,640
8	1,610	743	3,490
9	2,300	1,030	5,160
10	—	1,180	—

This Table may be expanded as follows for inocula greater or smaller than 0·1 ml of the sample.

No. of positive tubes	MPN (/100 ml) when vol. (ml) inoculated is			
	10·0	1·0	0·1	0·01
0	—	—	—	—
1	1·05	10·5	105	1,050
2	2·23	22·3	223	2,230
3	3·57	35·7	357	3,570
4	5·11	51·1	511	5,110
5	6·93	69·3	693	6,930
6	9·16	91·6	916	9,160
7	12·00	120	1,200	12,000
8	16·10	161	1,610	16,100
9	23·00	230	2,300	23,000
10	—	—	—	—

7. REFERENCES

ANDERSON, E. S. (1960). The occurrence of *Salmonella paratyphi* B in desiccated coconut from Ceylon. *Mon. Bull. Minist. Hlth Lab. Serv.* **19**, 172.

Christensen, W. B. (1946). Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. *J. Bact.* **52**, 461.

Cook, C. T. (1952). Comparison of two modifications of bismuth-sulphite agar for the isolation and growth of *Salmonella typhi* and *Salm. typhi-murium*. *J. Path. Bact.* **64**, 559.

Dixon, J. M. S. & Pooley, F. E. (1961). Salmonellae in a poultry processing plant. *Mon. Bull. Minist. Hlth Lab. Serv.* **20**, 30.

Galbraith, N. S., Hobbs, B. C., Smith, M. E. & Tomlinson, A. J. H. (1960). Salmonellae in desiccated coconut. *Mon. Bull. Minist. Hlth Lab. Serv.* **19**, 99.

Gillies, R. R. (1956). An evaluation of two composite media for preliminary identification of *Shigella* and *Salmonella*. *J. clin. Path.* **9**, 368.

Harvey, R. W. S. (1956). Choice of a selective medium for the routine isolation of members of the salmonella group. *Mon. Bull. Minist. Hlth Lab. Serv.* **15**, 118.

Harvey, R. W. S. & Thomson, S. (1953). Optimum temperature of incubation for isolation of salmonellae. *Mon. Bull. Minist. Hlth Lab. Serv.* **12**, 149.

Hobbs, B. C. & Allison, V. D. (1945). Studies on the isolation of *Bact. typhosum* and *Bact. paratyphosus* B. *Mon. Bull. Minist. Hlth Lab. Serv.* **4**, 63.

Hobbs, B. C. & Wilson, J. G. (1959). Contamination of wholesale meat supplies with salmonellae and heat-resistant *Clostridium welchii*. *Mon. Bull. Minist. Hlth Lab. Serv.* **18**, 198.

Houston, A. C. (1914). 10*th Rep. metrop. Water Bd.*

Jameson, J. E. (1961). A study of tetrathionate enrichment techniques, with particular reference to two new tetrathionate modifications used in isolating salmonellae from sewer swabs. *J. Hyg., Camb.*, **59**, 1.

Lendon, N. C. & Mackenzie, R. D. (1951). Tracing a typhoid carrier by sewage examination. *Mon. Bull. Minist. Hlth Lab. Serv.* **10**, 23.

McDonagh, V. P. & Smith, J. G. (1958). The significance of the abattoir in salmonella infection in Bradford. *J. Hyg., Camb.*, **56**, 271.

Memorandum (1961). Serological reagents for bacteriological diagnosis. Publ. Hlth Lab. Serv. Memorandum no. 4. *Mon. Bull. Minist. Hlth Lab. Serv.* **20**, 134.

Moore, B. (1950). The detection of typhoid carriers in towns by means of sewage examination. *Mon. Bull. Minist. Hlth Lab. Serv.* **9**, 72.

Murdock, C. R. (1954). The occurrence of salmonella in liquid egg. *Mon. Bull. Minist. Hlth Lab. Serv.* **13**, 43.

Report (1947). The bacteriology of spray-dried egg with particular reference to food poisoning. *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 260. London: H.M.S.O.

Report (1955). Salmonellae in liquid egg including frozen liquid egg. *Mon. Bull. Minist. Hlth Lab. Serv.* **14**, 65.

Report (1958). The contamination of egg products with salmonellae with particular reference to *Salm. paratyphi* B. *Mon. Bull. Minist. Hlth Lab. Serv.* **17**, 36.

Report (1959*a*). An examination of English egg products. *Mon. Bull. Minist. Hlth Lab. Serv.* **18**, 158.

Report (1959*b*). Salmonella organisms in animal feeding stuffs and fertilizers. *Mon. Bull. Minist. Hlth Lab. Serv.* **18**, 26.

Report (1959*c*). Sewage contamination of coastal bathing waters in England and Wales. *J. Hyg., Camb.*, **57**, 435.

Report (1961). Salmonella organisms in animal feeding stuffs. *Mon. Bull. Minist. Hlth Lab. Serv.* **20**, 73.

Rolfe, V. (1946). A note on the preparation of tetrathionate broth. *Mon. Bull. Minist. Hlth Lab. Serv.* **5**, 158.

Smith, J. G. (1959). Observations on the isolation of salmonellae from selenite broth. *J. appl. Bact.* **22**, 116.

Thomson, S. (1953). Paratyphoid fever and bakers' confectionery. *Mon. Bull. Minist. Hlth Lab. Serv.* **12**, 187.

Walker, J. H. C. (1957). Organic fertilizers as a source of salmonella infection. *Lancet* **ii**, 283.

Wilson, W. J. (1938). Isolation of *Bact. typhosum* by means of bismuth-sulphite medium in water- and milk-borne epidemics. *J. Hyg., Camb.*, **38**, 507.

Wilson, W. J. & Blair, E. M. McV. (1927). Use of a glucose-bismuth-sulphite-iron medium for the isolation of *B. typhosus* and *B. proteus*. *J. Hyg., Camb.*, **26**, 374.

8. EXPLANATION OF PLATES

PLATE 1. The effect of refrigeration on the inhibitory properties of bismuth-sulphite agar.

(a) No refrigeration, plate inoculated immediately after pouring. Inhibition very severe.
(b) Refrigerated for 24 hr before inoculation. Inhibition decreased.
(c) Refrigerated for 48 hr before inoculation. Inhibition slight.
(d) Refrigerated for 96 hr before inoculation. No inhibition.

All the plates are from the same batch and were similarly inoculated from the same 18 hr bismuth-sulphite culture of a salmonella. (All × ½).

PLATE 2. (a) and (b). The characteristic salmonella colony after 18 hr at 37° on bismuth-sulphite agar. (× 5).

(a) Phase 1. Where colonies are confluent, the continuation of the clear growing edge around the periphery is characteristic.

(b) Phase 2. The colonies are flat, the black centre is reduced and the translucent growing edge larger.

(c), (d) and (e). Colonies of organisms other than salmonella after 18 hr at 37° on bismuth-sulphite agar. (× 5).

(c) *Escherichia.*
(d) *Proteus.*
(e) *Proteus*, after a further 4 hr at room temperature to permit spreading.

(f) Enrichment of sewage subcultured on bismuth-sulphite agar, after 18 hr at 37°. (× 5).Characteristic salmonella colonies are recognizable on sight.

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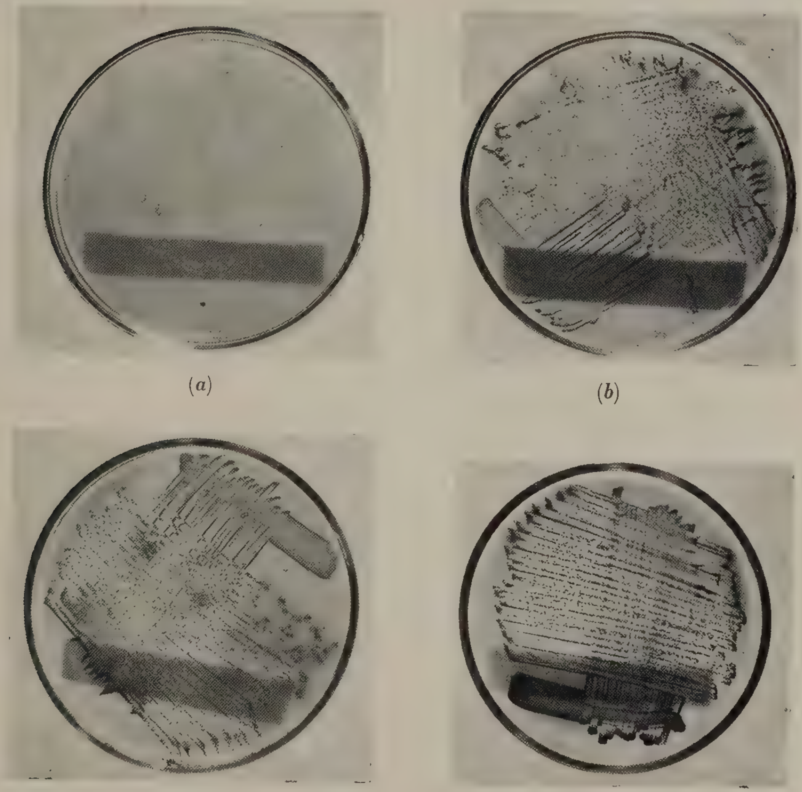
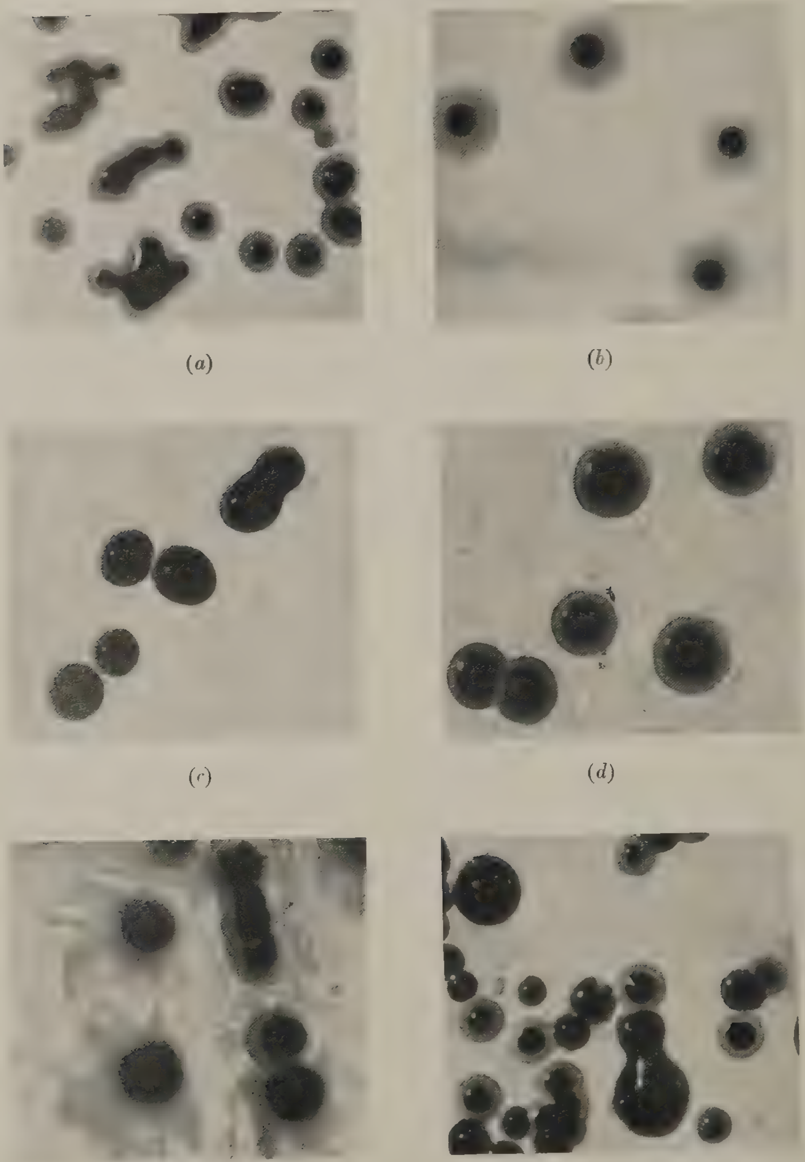


(*a*) (*b*)

(*c*) (*d*)

J. H. McCoy—The Isolation of Salmonellae. Plate 1



(*a*) (*b*)

(*c*) (*d*)

(*e*) (*f*)

J. H. McCoy—The Isolation of Salmonellae. Plate 2

CASH, J. D. & COLLEE, J. G. (1962). *J. appl. Bact.* **25** (2), 225–231.

SPORULATION AND THE DEVELOPMENT OF RESISTANCE IN SPORING CULTURES OF *CLOSTRIDIUM WELCHII*

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SUMMARY: The growth of a strain of *Clostridium welchii* type A in Ellner's medium was studied and the development of spores was correlated with the emergence of forms resistant to deleterious influences including heat, desiccation and thiomersalate. A qualitative increase in heat resistance was demonstrated, coincident with spore maturation over the course of 12 hr. The granular appearance of the bacterial cytoplasm prior to sporulation, as revealed by Gram's stain, precedes the development of acid-fast material and does not seem to be associated with the presence of sudanophilic or metachromatic material.

ALTHOUGH much interest has been shown in bacterial spores there is still much to be learned about the process of sporulation (Young & Fitz-James, 1959). With only a few exceptions, most of the significant investigations of sporogenesis have involved studies of aerobic sporeforming bacilli. Little is known, for example, of the sporulation process of *Clostridium welchii* because this organism does not readily form spores in laboratory media. However, *Cl. welchii* spores occur widely in nature and are potential causes of disease in man and animals. The classical strains of *Cl. welchii* type A associated with gas gangrene are similar to typical food poisoning strains, but the latter produce spores which are exceptionally heat resistant. The heat resistance of spores of food poisoning strains seems to be genetically determined and is not dependent on the ability of these strains to produce more spores than classical strains (Collee, Knowlden & Hobbs, 1961). *Cl. welchii* thus appears to provide a model for the detailed study of the phenomenon of heat resistance, if techniques for the investigation of its spores can be further developed.

Ellner (1956) devised a medium for promoting the rapid quantitative sporulation of *Cl. welchii*. Using this medium, Smith & Ellner (1957) demonstrated a sporulation cycle of the organism and noted the early development of the spore primordium ('forespore') by means of phase contrast microscopy and a safranin staining technique. They suggested that further studies might correlate structural and physiological changes in the sporulating bacterial cell, and the purpose of the work reported here was to confirm the observations of these authors and to discover whether progressive changes occur in the resistance of the developing spore of *Cl. welchii* to deleterious influences.

METHODS

General

Cultures. Sporing cultures of a strain of *Cl. welchii* type A (strain O32, isolated from a stab wound) were prepared by transferring 10 ml of the last of three serial

8–12 hr cooked meat broth subcultures to 50 ml of freshly steamed Ellner's medium at 37°. Serial subculture in cooked meat broth for a few hours prior to inoculation of the Ellner medium minimized the probability of transferring small numbers of spores, which may occur in older cooked meat broth cultures. The presence of spores in the vegetative culture used for inoculating the Ellner medium would have invalidated its use as a control in the resistance tests. Routine checks of purity were done by subculturing on to horse blood agar plates. The Ellner cultures were incubated anaerobically at 37° in an autocatalytic anaerobic jar (Baird & Tatlock, Ltd., Chadwell Heath, Essex, England) which could be opened and rendered anaerobic again within a few minutes.

Microscopic examination. Loopfuls of culture were taken at hourly intervals on to blocks of nutrient agar and impression smears were made from these on glass slides. The smears were stained by Gram's method and by a modified Ziehl-Neelsen stain, in which 2% (v/v) nitric acid in absolute alcohol was used for decolorization (Cruickshank *et al.* 1960, p. 121). Similar smears were stained with Sudan black to detect lipid inclusion granules and with Albert's stain for metachromatic granules (Cruickshank *et al.* 1960, p. 119). Wet films of culture were examined simultaneously by phase contrast microscopy, using an oil immersion (1/12 in.) objective.

Washed bacterial suspension. At hourly intervals, 5 ml amounts of Ellner culture were removed, added to 7 ml of sterile physiological saline and centrifuged at 3,000 rev/min for 10–15 min. After discarding the supernatant fluid the deposit was resuspended in 12 ml of sterile saline and centrifuged. The second supernatant was also discarded and the washed deposit finally resuspended in 5 ml of sterile saline.

There was some evidence of a factor protective against desiccation on organisms grown in cooked meat broth: this was eliminated by thorough washing.

Resistance tests

The results of numerous preliminary experiments to define the limits of resistance of the vegetative form of strain O32 indicated that it could not survive exposure to desiccation in air for a few minutes, moist heat at 60° for 10 min in unbuffered physiological saline, or 1:1,250 aqueous sodium thiomersalate solution ('merthiolate', sodium ethylmercurithiosalicylate) for 30 min at 20°. On the basis of these observations the following tests were developed.

Desiccation resistance test. A standard drop (0·02 ml) of washed bacterial suspension was spread with a loop over the entire surface (7·5 × 2·5 cm) of each of four sterile glass slides held under a sterilized metal canopy. Drying occurred within 5 min at room temperature (20°). After 1 hr the slides were inverted on to the surface of a plate of egg yolk medium (Willis & Hobbs, 1959) containing 100 μg of neomycin sulphate/ml to suppress contaminants. The slides were pressed to ensure contact and removed after 2 min. The impressed plates were incubated anaerobically at 37° for 20 hr and viable (survival) counts subsequently obtained by counting colonies.

Heat resistance tests. Washed bacterial suspension (0·5 ml) was transferred to a preheated thin glass ampoule which was then sealed in a blast flame and totally immersed in water at 60°. After 10 min the ampoule was cooled rapidly in ice and 4 drops (each of 0·02 ml) of the contents were pipetted on to Willis & Hobbs' medium for viable counts (Miles & Misra, 1938) after 20 hr incubation at 37°. This type of test was performed in duplicate for each hourly sample and identical procedures were undertaken at temperatures of 73° and 80°.

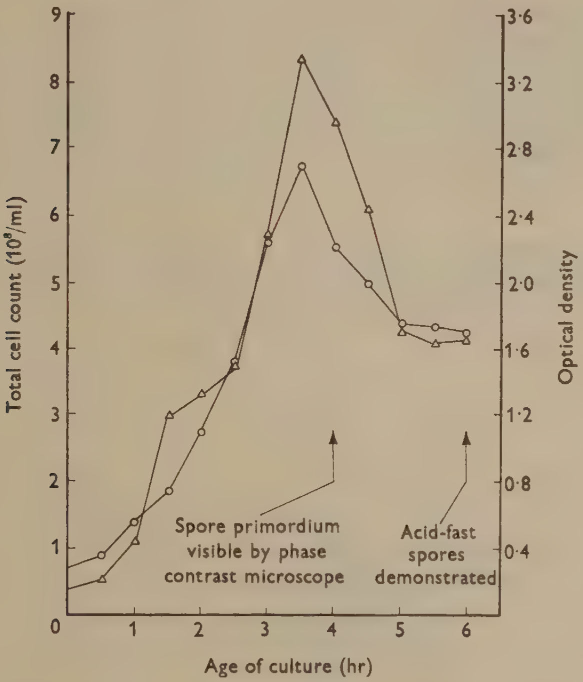

Fig. 1. The growth of *Cl. welchii* and the development of spores in Ellner's medium at 37°.
Triangles, total cell counts; circles, optical density (1 cm cell).

Thiomersalate resistance test. Washed bacterial suspension (0·5 ml) was thoroughly mixed with 2 ml of aqueous thiomersalate solution to give a final thiomersalate concentration of 1:1,250. After 30 min at 20°, 0·1 ml of the mixture was transferred to 9·9 ml of sterile saline and, immediately after mixing, four 0·02 ml drops were pipetted on to Willis & Hobbs' medium for viable counts as for the heat resistance tests described above. This procedure was duplicated for each hourly sample. The final dilution of thiomersalate (1:125,000) was shown to have no inhibitory effect on the growth of this strain of *Cl. welchii* on Willis & Hobbs' medium.

Survival. Viable counts were made on each hourly sample of washed bacterial suspension, and the counts obtained after exposing specimens of these samples to the resistance tests were expressed as percentages of the original viable counts.

When spores became numerous, initial dilutions of the washed suspensions were made prior to the tests in order to obtain reliable values.

Total cell counts. These were made with a Helber type counting chamber and phase contrast microscope.

RESULTS

Growth studies of this strain of *Cl. welchii* in Ellner's medium showed (Fig. 1) that the use of inoculum from young cooked meat broth cultures led to the virtual elimination of a lag phase. Fig. 1 also shows that growth proceeded exponentially for 5 generations (mean generation time 42 min) until maximum total cell counts and turbidity readings (1 cm cells) were obtained at 3½ hr. Thereafter, the bacterial counts and turbidity readings revealed a reduction in numbers coincident with the microscopic observation of lyzed forms and the initiation of sporulation. Then followed a stationary phase, when spore development continued but bacterial multiplication was not resumed. The microscopic observations are summarized in Table 1. First evidence of the development of the spore primordium in the Ellner cultures was obtained by phase contrast microscopy at 4 hr, some 2 hr before acid-fast spores could be demonstrated. Overnight Ellner cultures examined at 20 hr contained

Table 1. *A comparison of the features of* Cl. welchii *cultures in cooked meat broth and Ellner's medium observed in wet films by phase contrast microscopy and in films stained by a modified Ziehl-Neelsen method*

Medium	Time (hr)	Examination by	
		Phase contrast	Ziehl-Neelsen stain
Cooked meat broth	0*	Short, stout rods; homogeneous cytoplasm	Short, stout rods; no acid-fast material
Ellner	1	Rods elongating; cytoplasm mottled	Rods elongating; no acid-fast material
	2	Continued elongation of rods; cytoplasm mottled	Elongated rods; no acid-fast material
	3	Rods shorter and stouter. Some apparently lyzed 'ghost forms' seen	Rods shorter and granular; no acid-fast material
	4	First evidence of faint subterminal condensations in bacterial cytoplasm (about 1/500 organisms)	Granular appearance more marked; no acid-fast material
	5	Many forespore forms (about 1/10 organisms)	Granular appearance reduced; no acid-fast material
	6	Forespores in 1/5 organisms and refractile spores in 1/100 organisms	First evidence of acid-fast spores in about 3/500 organisms
	7	Greatly increased numbers of refractile spores. Forespores still seen in about 1/100 organisms	Acid-fast spores now number about 1/60 organisms
	8	Free spores present	Free acid-fast spores present

* Young vegetative cells used as inoculum.

very numerous free spores, of which many were frequently found to be impermeable to the Ziehl-Neelsen stain, being seen in outline as regular oval structures considerably larger than the acid-fast spores. Staining by Sudan black and Albert's stain revealed no intracellular accumulations of sudanophilic or metachromatic material in the vegetative cells either preceding or during the sporulation process.

Development of resistance. The results of the resistance tests are shown in Table 2. Forms resistant to heat at 60° were first detected in the Ellner cultures at 4 hr. Desiccation resistance was demonstrable at 5 hr and thiomersalate resistance at 6 hr. Thereafter the percentage survival increased with time in the heat resistance and desiccation resistance tests. There was no quantitative increase in thiomersalate resistance after the initial detection of forms resistant to thiomersalate. A truly qualitative increase in heat resistance was demonstrated in a separate series of tests at 60°, 73° and 80° (Table 3).

Table 2. *The effect of age of culture on the survival of washed suspensions of Ellner cultures of* Cl. welchii *exposed to various deleterious influences*

Nature of stress	Survival (%) in cultures aged (hr)						
	0	1	4	5	6	7	8
Heat at 60° for 10 min	0	0	0·003	1·56	4·6	17·2	24·4
Desiccation at 20° for 60 min	0	0	0	0·02	0·50	0·71	8·01
Thiomersalate at 1:1,250 for 30 min	0	0	0	0	0·05	0·05	0·05

Table 3. *The effect of age of culture on the survival of washed suspensions of Ellner cultures of* Cl. welchii *exposed for* 10 *min to different temperatures*

Temp. (°C)	Survival (%) of cultures aged (hr)								
	0	1	3	4	5	6	7	8	12
60	0	0	0	0·1	0·9	7·8	18·4	36·7	67·0
73	0	0	0	0	0	0·003	2·8	10·4	31·6
80	0	0	0	0	0	0	0·0003	0·0004	0·0018

DISCUSSION

The morphological development of spores of *Cl. welchii* observed in the Ellner cultures is similar to that described by Smith & Ellner (1957). It has been possible to correlate the changes in structural appearance with the resistance of the developing spore to deleterious influences, the detection of resistant forms coinciding with the microscopic observation of the initiation of sporogenesis. Smith & Ellner noted the occurrence of encystment of *Cl. welchii* forespores in Ellner cultures within 4–5 hr and that this was associated with increased resistance of these structures to hydrolysis. Young & Fitz-James (1959) noted forespore development in cultures of *Bacillus cereus* $1\frac{1}{2}$ hr after cessation of growth. In the present investigation, forespore development in *Cl. welchii* was first observed by phase contrast microscopy at 4–5 hr, i.e. $\frac{1}{2}$–1$\frac{1}{2}$ hr after cessation of growth, and this coincided with the emergence of heat resistant forms. The granular appearance of the bacterial cytoplasm, revealed by Gram's stain and seen also by phase contrast microscopy or by Ziehl-Neelsen staining prior to sporulation, preceded the development of acid-fast material. The negative results obtained with the Sudan black and modified Neisser (Albert's) stains suggest that the granular appearance of the stained cytoplasm is not attributable to deposits of sudanophilic or metachromatic material, unless these are present in a form which does not react with the stains used. Acid-fast spores were not demonstrable until 6 hr. At this stage, Smith & Ellner first encountered forespores resistant to the mordanting effect of potassium permanganate.

A quantitative and a qualitative increase in the development of heat resistant forms in parallel with spore maturation was clearly demonstrated in the present study and this was progressive for the observation period of 12 hr. Thus, refractile spores were first noted at 6 hr, but the evidence of the heat resistance tests indicates that spore maturation proceeded for at least a further 6 hr, i.e. for 8$\frac{1}{2}$ hr after cessation of growth. With *B. cereus*, Young & Fitz-James demonstrated a period of 5–6 hr between the cessation of vegetative division and the emergence of refractile spores. The slow development of significant heat resistance of *Cl. welchii* spores in the present study is consistent with the general impression that young refractile spores which are acid-fast are not necessarily 'mature' as regards heat resistance. The lack of any quantitative increase in thiomersalate resistance after the initial detection of forms resistant to the antiseptic may be partly explained on the basis that adsorption of thiomersalate to sporing organisms prevented subsequent dilution of the antiseptic and resulted in the retention of lethal concentrations at germination: but this explanation is not wholly satisfactory.

Young & Fitz-James discussed the dangers of making observations on a whole culture to determine a sequence of events associated with spore formation in a single bacterium; if observations on whole culture were to be valid, there should be no further growth in the culture after sporulation and there should be a reasonable degree of synchrony in the process. That these criteria have been partially met in the present work is perhaps fortunately due to the growth pattern of *Cl. welchii* in Ellner's medium, but overlapping of the various stages in spore maturation is evident. Attempts to produce synchronous Ellner cultures would probably yield more significant results. Although the simple techniques described in the present investigation do not allow for such variables as failure of viable spores to germinate and inaccuracies involved in making impression cultures from slides, difficulty in obtaining reproducible results was mainly encountered in securing consistently high degrees of sporulation in Ellner's medium. Despite intensive efforts no reliable solution was found to this problem, which greatly increased the work involved in the investigation. The results obtained nevertheless endorse the view of Smith & Ellner (1957) that the sporulation process of *Cl. welchii* may be a useful model for further cytochemical and metabolic studies, and indicate the need for further development of a sporulation medium for *Cl. welchii*.

The authors wish to thank Dr. J. P. Duguid for his helpful advice.

REFERENCES

COLLEE, J. G., KNOWLDEN, J. A. & HOBBS, B. C. (1961). Studies on the growth, sporulation and carriage of *Clostridium welchii* with special reference to food poisoning strains. *J. appl. Bact.* **24**, 326.

CRUICKSHANK, R. *et al.* (1960). Mackie & McCartney's *Handbook of Bacteriology*, 10th ed. Edinburgh: E. & S. Livingstone.

ELLNER, P. D. (1956). A medium promoting rapid quantitative sporulation in *Clostridium perfringens*. *J. Bact.* **71**, 495.

MILES, A. A. & MISRA, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.*, **38**, 732.

SMITH, A. G. & ELLNER, P. D. (1957). Cytological observations on the sporulation process of *Clostridium perfringens*. *J. Bact.* **73**, 1.

WILLIS, A. T. & HOBBS, G. (1959). Some new media for the isolation and identification of clostridia. *J. Path. Bact.* **77**, 511.

YOUNG, I. E. & FITZ-JAMES, P. C. (1959). Chemical and morphological studies of bacterial spore formation. I. The formation of spores in *Bacillus cereus*. *J. biophys. biochem. Cytol.* **6**, 467.

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Cooper, R. (1962). *J. appl. Bact.* **25** (2), 232–236.

THE RETENTION OF ^{32}P-LABELLED *RHIZOBIUM* BY LEGUME SEED AFTER INOCULATION BY VACUUM TREATMENT

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SUMMARY: Legume seeds were vacuum treated in a suspension of ^{32}P-labelled *Rhizobium meliloti* to find the numbers and uniformity of distribution of bacteria taken up and retained by the seeds after washing. Commercial seed retained 5–12 times as many bacteria as did hand harvested seed. From 5 to 15% of the seeds held 75–90% of the bacteria; vacuum treatment increased uptake by this small group of seeds by 60–300%, but had no significant effect on the majority that acquired relatively few bacteria. Therefore vacuum treatment of seeds during inoculation is likely to affect nodulation on a small proportion of the plants only.

There is conflicting evidence whether alternately applying a vacuum and atmospheric pressure to legume seed during its inoculation with *Rhizobium* (Porter, McAlpine & Kaerwer, 1960) increases the proportion of nodulated plants produced and promotes survival of the inoculum on stored seed. Loneragan, Moye & Anderson (1961) obtained more nodulated plants from vacuum inoculated seeds than from seeds inoculated in the conventional manner, all seeds being stored 12–52 weeks before sowing. However, when they sowed vacuum inoculated and stored seeds in pairs on agar slopes in test tubes, only 8–42% of the plants nodulated, suggesting that 60–95% of the seeds were essentially free from living *Rhizobium* after storage.

The experiments described below were designed to find how vacuum treatment affects the numbers of bacteria taken up by different samples of legume seed and how they are distributed between the seeds.

Methods

Rhizobium meliloti, strain AH2, was grown on the following low phosphate medium to produce a high bacterial content of ^{32}P: sucrose, 10 g; yeast extract (Difco), 2 g; $MgSO_4.7H_2O$, 100 mg; $CaCl_2$, 25 mg; K_2SO_4, 50 mg; K_2HPO_4, 5 mg; carrier-free solution of $H_3{}^{32}PO_4$, 8 mc; distilled water, 1 litre. Medical flat bottles (3 oz) containing 20 ml of this medium were sterilized by autoclaving at 10 lb/in^2 for 15 min, and inoculated with a loopful of culture grown for 2 days on the same medium but with the $H_3{}^{32}PO_4$ and the K_2SO_4 omitted and the K_2HPO_4 increased to 500 mg/litre.

The bottles were incubated face downwards at 30° for 2 days, when the bacteria were centrifuged, washed 4 times with 1% (w/v) saline and finally resuspended in a volume of 1% saline equal to the original volume of culture. Such suspensions contained about 10^9 bacteria/ml and 60–70% of the ^{32}P originally added to the growth medium, after allowing for radioactive decay. Leakage of the bacterial ^{32}P

into the surrounding medium, which amounted to 3–4% in 1 hr, was unaffected by the vacuum treatments.

Batches of 200 undamaged and hand selected seeds of the legumes named in Table 1, in loosely screwcapped bottles, were each immersed in 2·5 ml of either the washed suspensions or the recentrifuged culture fluid (to estimate the uptake of ^{32}P compounds in solution), together with 0·1 ml of 1% (v/v) 'Byprox' (an anionic detergent) to help wet the seeds. A vacuum desiccator containing the bottles was shaken gently for 45 min while the pressure was 3 times lowered to 20 mm of Hg and suddenly restored to 750 mm of Hg; controls were shaken without vacuum treatment.

Seeds were shaken and washed, usually 4 times, with 50 ml of water over a period of 2 hr, dried at 30°, and stuck separately to glass plates with a strong glue. Sheets of Kodak 'Microtex' X-ray film were applied closely to the seeds, exposed for 4 days, and examined for dark spots coincident with the seeds. The more radioactive seeds were then digested individually by boiling for 3–5 min with 0·5 ml of conc. H_2SO_4; the others were bulked before digestion. The resulting fine, black suspensions were made up to 10 ml and their radioactivity measured with a Mullard type MX124 liquid counter and a Panax type SA102 scaler. Corrections were made for the background activity of untreated seeds digested as above, for decay of the ^{32}P, and for the small amount of ^{32}P which the seeds acquired directly from the suspending fluid contaminated by leakage from the bacteria. Aliquots from the bacterial suspensions were similarly digested and counted, and the bacterial concentration measured, to give the radioactivity per bacterium and hence the number of bacteria per seed.

For germination tests, further batches of 200 untreated seeds were incubated on wet filter paper for 24 hr at 0–4° and then for 7 days at 25° (for lucerne and red clover) or at 18° (for subterranean clover). Germinating seeds, seeds imbibing but not germinating, and seeds remaining hard, were counted.

RESULTS

The number of bacteria retained and the increased retention from vacuum treatment depended on the type of seed (Table 1). The commercial seed held many more bacteria than did the hand harvested samples, of which more remained hard in the germination tests. Within the pairs of samples of lucerne and red clover, the sample which germinated the better held the fewer bacteria.

On the autoradiograms of both vacuum treated and control seeds (Plate 1), an occasional very dark spot or line coincided with a hole or crack in the seed coat, but high radioactivity was not consistently associated with any macroscopic appearance of the seeds; nor were holes or cracks consistently associated with high radioactivity.

Plate 1 illustrates how very unevenly the bacteria were distributed between individual seeds; 5–15% of the seeds held 75–95% of the bacteria. In constructing Table 2, the seeds were divided arbitrarily into two groups according to whether they held more or fewer than 2×10^5 bacteria. Vacuum treatment increased the number held by the first group but not, except with the hand harvested red clover, by the second, which held relatively few bacteria whether or not vacuum treated. Vacuum treatment made distribution more uneven in all samples.

Table 1. *The average retention* of bacteria by seeds of different percentage germination immersed in a suspension of* Rhizobium meliloti† *and washed* 4 *times*

Seed sample	Germination of imbibed seed (%)	Amount of hard seed (%)	Bacteria on seed (10^3/seed)	
			Not vacuum treated	Vacuum-treated
Lucerne (commercial 1)	76	0·3	50	57
Lucerne (commercial 2)	96	0	27	37‡
Red clover (commercial)	29	1	23	41‡
Red clover (hand harvested)	64	6	4·1	14‡
Subterranean clover (hand harvested)	63	54	3·6	5·6‡

* Means of results from duplicate batches. † Concentration, 10^9 bacteria/ml.
‡ The increase due to vacuum treatment was significant at $P < 0{\cdot}05$.

Table 2. *The distribution of bacteria within batches* of* 200 *seeds immersed in a suspension of* Rhizobium meliloti† *and washed* 4 *times, according to bacterial load/seed*

Seed sample	Vacuum treatment	Nos. of seeds (a) and total bacteria (b)				Total bacteria‡ on all 200 seeds
		$<2\times10^5$/seed		$>2\times10^5$/seed		
		a	b‡	a	b‡	
Lucerne (commercial 1)	−	190	3·0	10	7·1	10·1
	+	190	2·2	10	9·2§	11·4
Lucerne (commercial 2)	−	191	2·6	9	2·8	5·4
	+	191	1·4	9	6·1§	7·5§
Red clover (commercial)	−	194	0·90	6	3·8	4·7
	+	189	1·07	11	7·2§	8·3§
Red clover (hand harvested)	−	199	0·62	1	0·21	0·83
	+	195	1·11§	5	1·80§	2·9§
Subterranean clover (hand harvested)	−	200	0·72	0	0·00	0·72
	+	199	0·55	1	0·56§	1·11§

* Means of results from duplicate batches. † Concentration, 10^9 bacteria/ml. ‡ In millions.
§ The increase due to vacuum treatment was significant at $P < 0{\cdot}05$.

The average bacterial uptake by lucerne seeds was positively correlated with the density of the suspension in which they were immersed, with or without vacuum treatment (Fig. 1).

In a separate experiment, further washings successively removed bacteria from lucerne seed (Fig. 2); the effect of vacuum treatment disappeared after the seed had been washed between 10 and 20 times.

Compounds in the recentrifuged culture fluid which contained ^{32}P were also taken up by the lucerne seed, and the effects of vacuum treatment and washing were similar to those for ^{32}P-labelled bacteria (Fig. 2). The volume of seed inoculant containing the average amount of ^{32}P retained by one vacuum treated seed after 4 washes was $4{\cdot}5 \times 10^{-5}$ ml for bacteria and $7{\cdot}5 \times 10^{-5}$ ml for ^{32}P-compounds in solution. Thus the proportion transferred to the seeds from a given volume of fluid was of the same order for bacteria as for ^{32}P-compounds in solution.

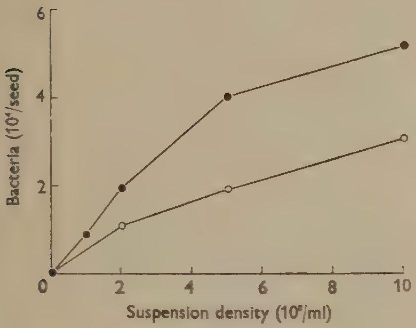

Fig. 1

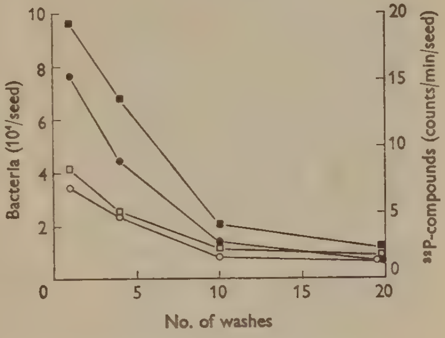

Fig. 2

Fig. 1. The effect of density of bacterial suspension on the average retention of *Rhizobium meliloti* by 200 lucerne seeds (commercial sample no. 2) after 4 washes.
Full circles, vacuum treated; open circles, not vacuum treated.

Fig. 2. The effect of vacuum treatment and washing on the average retention by 200 lucerne seeds (commercial sample no. 2) of *Rhizobium meliloti* (from a suspension of 10^9 bacteria/ml) or of ^{32}P-compounds (from a solution of 180,000 counts/min/ml).
Full circles, bacteria, vacuum treated: open circles, bacteria, not vacuum treated: full squares, ^{32}P-compounds, vacuum treated: open squares, ^{32}P-compounds, not vacuum treated.

DISCUSSION

The methods described here probably assess fairly the usefulness of vacuum treatment, although more bacteria will be removed from the seeds by washing than by the commercial technique of drying the seeds directly from the inoculant. The washing allowed direct estimation of the bacteria held under the seed coat or within its surface irregularities, and therefore probably magnified the percentage increase in bacterial numbers caused by vacuum treatment. If the bacteria survive better within or underneath the seed coat, as supposed by Porter *et al.* (1960), then washing the seed should leave behind the bacteria with the best chance of nodule initiation. However, in all samples such bacteria were very unevenly distributed between different seeds. This may be explained by two further observations. First, the commercial seed retained many more bacteria than did the hand harvested seed, possibly because the commercial seed was scarified; Porter *et al.* (1960) recommended that hard coated seeds should be scarified before inoculation. Second, for a given host species, the samples which germinated better held the fewer bacteria. Thus, the seeds which held many bacteria were probably those with damaged coats and perhaps included many of the nongerminaters. There was only partial correlation between visible signs of damage to the seed coats and high bacterial numbers, but significant damage was probably often microscopic.

Bacteria behaved like dissolved compounds of phosphorus in the effects produced by vacuum treatment and washing of the seeds, and in the proportion taken up from a given volume of inoculant fluid. Thus the bacteria were probably taken up merely as components of the suspending fluid rather than by any more specific attachment to the seeds.

The main conclusion from this work is that the small proportion of nodulating plants produced by seed stored after vacuum inoculation with *Rhizobium* (Loneragan *et al.* 1961) probably reflects the small proportion of the seeds that takes up large numbers of bacteria. Vacuum treatment may increase the chances of nodulation by plants from this small group of probably damaged and perhaps poorly germinating seeds, but has little effect on the great majority of the seeds.

The author wishes to thank Dr. P. S. Nutman for helpful suggestions and discussion, and Mrs. L. Longbone for technical assistance.

REFERENCES

LONERAGAN, J. F., MOYE, D. V. & ANDERSON, A. J. (1961). Survival of *Rhizobium* on clover seeds. *Nature, Lond.*, **192**, 526.

PORTER, F. E., MCALPINE, V. W. & KAERWER, H. E. (Jr.) (1960). Seed impregnation including bacterial and vacuum treatment. U.S. Pat. no. 2932128.

EXPLANATION OF PLATE

Autoradiograms of batches of 200 legume seeds inoculated with ^{32}P-labelled *Rhizobium meliloti* and washed 4 times. All × ½.

- (a) and (b). Lucerne (commercial sample 2).
  - (a) Not vacuum treated.
  - (b) Vacuum treated.
- (c) and (d). Red clover (hand harvested).
  - (c) Not vacuum treated.
  - (d) Vacuum treated.

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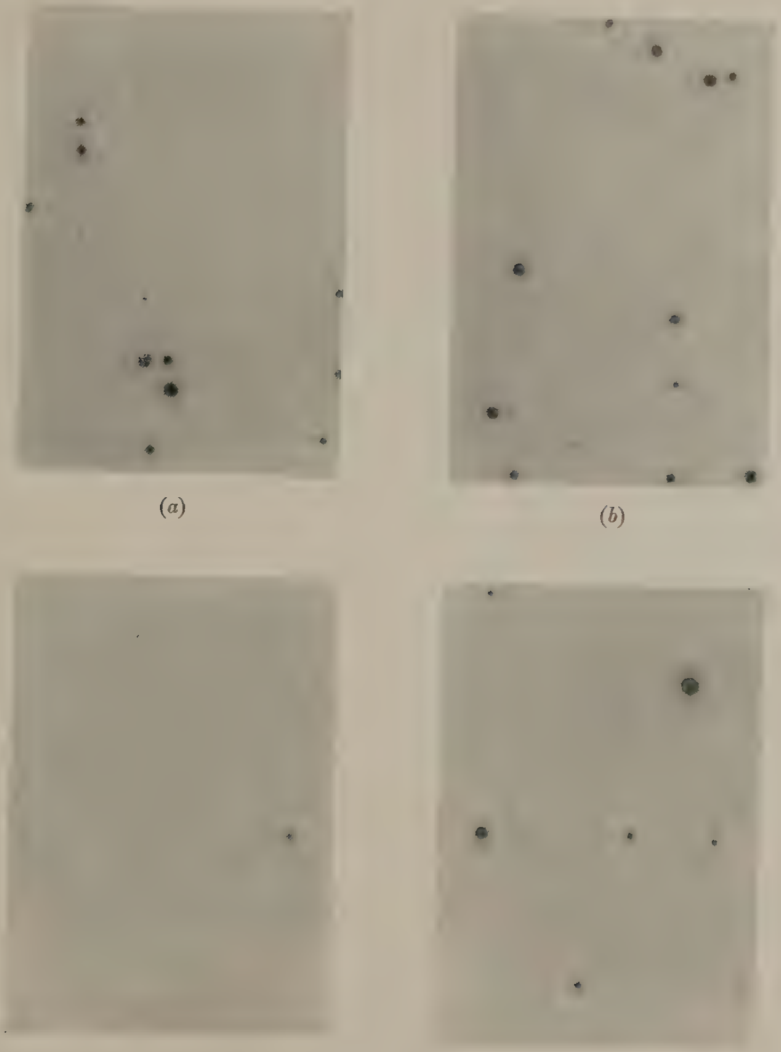

(*a*) (*b*)

(*c*) (*d*)

R. Cooper.—The Retention of ^{32}P-Labelled *Rhizobium* by Legume Seed after Inoculation by Vacuum Treatment. Plate 1

Eddy, B. P. & Ingram, M. (1962). *J. appl. Bact.* **25** (2), 237–247.

THE OCCURRENCE AND GROWTH OF STAPHYLOCOCCI ON PACKED BACON, WITH SPECIAL REFERENCE TO *STAPHYLOCOCCUS AUREUS*

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SUMMARY: *Staphylococcus aureus* occurs and survives in bacon curing brines and may be found on shop sliced and on vacuum packed bacon. Under anaerobic conditions it competes with the indigenous flora of the bacon: its growth is favoured by low numbers of other organisms and by high storage temperatures. Packs of bacon for sale should carry the instruction to keep in a cool place.

Changes in the packaging of foods, or in other marketing procedures, raise new problems of bacteriological control, and it has already been suggested (Ingram, 1960) that staphylococci might be more apt to grow on vacuum packed than on normal bacon. The present paper is a further consideration of this question.

There is no certainty that *Staphylococcus aureus* occurs at all on bacon, though it seems likely that this product might become contaminated during the handling it receives before curing. According to Hodge (1960), *Staph. aureus* is so ubiquitous that its occurrence on handled foods can hardly be avoided. There have already been reports that it occurs in curing brines (Buttiaux & Moriamez, 1958), where it might be expected to survive.

No case of food poisoning attributable to the presence of staphylococcal enterotoxin in bacon has been recorded in the United Kingdom. However, this may have been due to any one, or a combination, of several reasons. First, enterotoxin producing staphylococci may always have been absent: although this seems unlikely, none was found by Kitchell (1958). Second, they may have been present but have been unable to develop sufficiently to constitute a hazard: possibly a combination of temperature and aerobic conditions favoured the growth of the normal flora of the bacon, present in much larger numbers than the adventitious pathogens, or the time between contamination and consumption was too short. Third, the food poisoning staphylococci may have been able to grow but, because of the conditions (e.g. the salt or nitrite contents), were unable to produce enterotoxin. In the absence of recorded outbreaks of poisoning there is no information about the occurrence and behaviour of potentially enterotoxic staphylococci on bacon.

With vacuum packed bacon, however, the conditions are rather different from normal and it may be that in certain circumstances a vacuum pack of bacon would provide conditions favouring the growth of pathogenic staphylococci. This is particularly true with regard to temperature (Ingram, 1960). In spite of instructions to keep in a cool place (which are in any case not printed on all packs), it is expected that vacuum packed bacon will sometimes be subjected to too high a temperature for too long a time (cf. canned hams; Ingram & Hobbs, 1954).

The objects of the present investigation were accordingly as follows: first, to find whether food poisoning strains of *Staph. aureus* occur and can survive in curing brines; second, to see whether they actually occur on normal bacon and on vacuum packed bacon; third, to investigate the ability of these staphylococci to grow under the anaerobic conditions to be expected in a vacuum pack (cf. Ingram, 1962).

Lacking a suitable method of testing for the enterotoxin, we have not confirmed the possibility of its production under such conditions: this has, however, just been done by Thatcher, Robinson & Erdman (1962).

The effects of storage temperature and time on the general bacterial flora, and other properties, of commercial vacuum packed bacon will be the subject of separate communications.

METHODS

Media and incubation temperatures. The first problem to be faced was the choice of a procedure for isolating and counting coagulase positive staphylococci on bacon. When this work was done four selective media were in common use: mannitol-salt agar (Chapman, 1945), staphylococcus medium no. 110 (Chapman, 1946), Chapman-Stone medium (Chapman, 1948), and tellurite-glycine agar (Zebowitz, Evans & Niven, 1955) or modifications of it. The first three media depend largely for their selectivity on the ability of pathogenic staphylococci to grow in the presence of 7·5% of NaCl (Koch, 1942), or of 5·5% of NaCl plus 7·5% of ammonium sulphate, and to ferment mannitol: but salt is clearly of no value in the detection of pathogenic staphylococci when they are mixed with much larger numbers of salt tolerant bacon micrococci. The last medium depends upon the selective action of glycine, tellurite and lithium chloride: it is, however, at least partially inhibitory to some coagulase positive staphylococci (Sevel & Plommet, 1960). It was suggested earlier (Ingram, 1960) that a promising procedure might be the use of phenolphthalein phosphate (PP) agar (Barber & Kuper, 1951) incubated at 44° to eliminate 'curing micrococci'; but it was found that, with bacon, a high proportion of the phosphatase positive colonies were coagulase negative, so that a coagulase test is also necessary to complete the diagnosis.

Accordingly, as a preliminary to the present investigation, mannitol-salt (MS) agar (Oxoid), medium no. 110 (Oxoid), tellurite-glycine (Z) agar and phenolphthalein phosphate (PP) agar were compared. Comparative surface counts were made at both 37° and 44° of broth cultures of four strains of *Staph. aureus* (NCTC 7415, 7447, 8342, 8532) on these media with and without the addition of an unsterilized homogenate of bacon from a vacuum pack. The results are shown in Table 1. The tellurite-glycine agar gave very erratic results and was at once rejected. On the other hand there seemed to be little difference between the other media. Since PP agar is not a selective medium, unlike MS and 110, bacon and brine samples (see later) were plated on it and four representative coagulase negative phosphatase positive colonies were selected. These were then grown in nutrient broth and counted on PP, MS and 110 agar incubated at 37° and 44°. Four phosphatase negative (but

Voges-Proskauer positive) strains, i.e. *Staph. saprophyticus* of Shaw, Stitt & Cowan (1951), were treated similarly, and the results are shown in Table 2. Both the selective media gave erratic results, and all the colonies on the MS agar at both temperatures were mannitol positive.

Table 1. *A comparison of counts of broth cultures of four strains of* Staphylococcus aureus *on four media, with and without unsterilized bacon homogenate*, incubated at 37° and 44°*

N.C.T.C.‡ strain of *Staph. aureus*	Log$_{10}$ (colonycount/ml) obtained in the presence (+) and absence (−) of bacon homogenate* at															
	37°, on media†								44°, on media†							
	PP		MS		110		Z		PP		MS		110		Z	
	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
7415	6·84	6·82	6·85	6·85	7·11	6·84	<3·3	<3·3	7·11	6·90	6·70	6·71	7·04	6·85	<3·3	<3·3
7447	6·83	6·84	6·68	6·36	6·97	6·88	6·75	6·61	6·89	6·91	6·81	6·67	6·91	7·03	4·48	4·26
8342	6·73	6·73	6·75	6·15	6·75	6·91	6·81	6·90	7·00	6·85	6·64	6·48	6·93	6·90	<3·3	<3·31
8532	6·86	7·00	6·78	6·98	6·94	6·93	6·68	6·93	6·88	7·02	6·79	6·93	6·96	7·00	3·38	<3·3

* The homogenate itself contained negligible numbers of bacteria viable at 37° or 44°.

† Media: PP, nutrient digest agar+0·01% phenolphthalein phosphate: MS, mannitol-salt agar; 110, staphylococcus medium no. 110; Z, tellurite-glycine agar.

‡ N.C.T.C., National Collection of Type Cultures.

Table 2. *A comparison of counts of broth cultures of eight staphylococci, isolated at 44°, on three media incubated at 37° and 44°*

Strain no.	Phosphatase reaction	Log$_{10}$ (colony count/ml) obtained at					
		37°, on media*			44°, on media*		
		PP	MS	110	PP	MS	110
28	+	8·77	8·51	<1·6	8·77	8·60	<1·6
46	+	8·56	8·44	<1·6	8·58	8·49	<1·6
92	+	8·74	8·62	8·10	8·72	8·62	<1·6
279	+	9·08	<1·6	<1·6	9·03	<1·6	<1·6
142	−	9·32	9·05	9·05	8·98	8·91	8·97
145	−	8·83	8·37	<1·6	8·67	8·40	<1·6
173	−	8·75	<1·6	<1·6	8·75	<1·6	<1·6
189	−	8·64	<1·6	<1·6	8·64	<1·6	<1·6

* Media: PP, nutrient digest agar+0·01% phenolphthalein phosphate; MS, mannitol-salt agar; 110, staphylococcus medium no. 110.

It was clear, therefore, that both MS agar and 110 agar, while giving satisfactory counts of coagulase positive colonies, would give unreliable counts with coagulase negative staphylococci. In addition to the detection of *Staph. aureus*, we were concerned with the general spoilage pattern of bacon at different temperatures and were therefore interested in all the bacteria able to grow. In particular we felt that some value could be attached to counts of coagulase negative staphylococci since, especially if they were phosphatase positive, they could be considered as 'presumptive *aureus* counts' indicating that *Staph. aureus* might perhaps have grown had it been present on the bacon. Hence it was decided that most information would be obtained by using PP agar incubated at 44° as a routine plating procedure, phosphatase positive colonies being tested for coagulase.

Counting methods. Bacterial counts were made by homogenizing 50 g of bacon with 200 ml of sterile water and by surface plating, in duplicate, 0·025 ml of serial tenfold dilutions of the 1:5 homogenate; the first dilution was usually the lowest used and, in this case, the minimum number of bacteria which could be counted was 2×10^3/g. In experiments on storage—towards which this work was directed—the primary interest lay in looking for the presence of *Staph. aureus* in numbers so large that growth during storage might be presumed. Hence it was considered that a plating method, although unable conveniently to show numbers less than 2×10^3/g, would be adequate for the purpose.

In other experiments a modified dilution counting technique (most probable numbers, MPN) was used. The homogenate, and tenfold dilutions of it, were added either to triplicate tubes of Robertson's cooked meat medium containing 8% (w/v) of NaCl and incubated overnight at 44°, or to the same medium containing also sodium azide (1:6,000; Richards, Soulides & Soulides, 1945) and incubated at 37°. The contents of tubes showing growth were plated on PP agar and phosphatase positive colonies were tested for coagulase. In this way it was possible to obtain minimum counts of about 5/g. Similar results were obtained with the two media.

Further experimental details are given where necessary in the text.

RESULTS

Survival and occurrence of Staph. aureus *in curing brines*

The organisms used in this study of survival were two enterotoxic strains (57/8733 and 58/7779) and two nonenterotoxic strains (55/3226 and 55/3643). A sample of cover brine from a bacon factory was counted on PP agar at 37°, and 100 ml volumes of it were then inoculated with washed suspensions of the staphylococci to give counts approximately 1,000 times that of the indigenous 37° flora. Samples of brine inoculated with each organism, and uninoculated control samples were stored at 1°, 5° and 10° and counted at intervals on PP agar incubated at 44°. The results (Table 3) show that survival of the staphylococci was good at all temperatures and was better the lower the storage temperature.

Table 3. *The survival of coagulase positive staphylococci in an average bacon brine, stored at three temperatures*

Duration of storage (days)	$\log_{10}$ (phosphatase positive colony count/ml)* of											
	Enterotoxic strain no.						Nonenterotoxic strain no.					
	57/8733, at			58/7779, at			55/3226, at			55/3643, at		
	1°	5°	10°	1°	5°	10°	1°	5°	10°	1°	5°	10°
0	5·92	—	—	5·35	—	—	6·08	—	—	5·98	—	—
2	5·97	6·24	5·99	6·01	6·13	6·17	6·20	6·04	6·09	6·16	6·03	6·06
7	6·05	6·11	5·78	5·98	6·05	5·83	6·05	6·12	6·12	5·96	5·86	5·86
15	5·90	5·19	5·56	5·92	5·80	4·17	5·99	5·69	5·09	5·79	5·92	4·34
24	5·86	5·48	4·97	5·68	5·32	5·34	4·31	5·61	4·74	6·11	5·52	4·82
31	5·86	5·80	3·49	5·75	5·39	5·11	5·87	5·47	3·49	5·89	5·41	4·33
80	6·26	5·31	4·30	5·11	5·39	4·78	5·64	5·06	5·44	6·05	4·98	4·84

* Counts: on PP agar at 44°.

The occurrence of staphylococci was investigated in samples of storage brine obtained from eight bacon factories. Viable counts were made on PP agar at 37° and 44°. In addition, 1 ml of each brine was added to 10 ml of nutrient broth and incubated overnight at 37° and 44°; then viable counts on PP agar at 37° and 44° were made again. The results are in Table 4. Brine 3 failed to show any phosphatase positive colonies. The presence of phosphatase positive organisms in 1 ml was demonstrated for all the other brines by the enrichment technique, but the numbers (except in brine 1) must have originally been less than 50/ml. Coagulase positive strains were isolated from brine 1 at 37° and 44°, brine 2 at 44°, brine 5 at 37°, brine 7 at 44° and brine 8 at 37° and 44°. Only in the case of brine 5 were coagulase positive colonies found at 37° but not at 44°.

Table 4. *A comparison of bacterial counts on phenolphthalein phosphate agar of bacon brines, undiluted and after dilution with nutrient broth *and incubation for 24 hr at 37° and 44°*

Brine sample no.	Incubation temp. of plates (°C)	Log$_{10}$ (colony count/ml) of total organisms (a) and phosphatase positive organisms (b)					
		Undiluted brine		Brine-broth, 37°		Brine-broth, 44°	
		(a)	(b)	(a)	(b)	(a)	(b)
1	37	3·85	1·74	8·40	6·89	8·51	6·92
	44	2·30	<1·60	8·32	6·76	8·44	7·08
2	37	3·02	<1·60	8·19	<1·60	8·56	<1·60
	44	1·60	<1·60	7·90	5·72	8·95	<1·60
3	37	2·44	<1·60	7·58	<1·60	8·30	<1·60
	44	<1·60	<1·60	6·97	<1·60	8·29	<1·60
4	37	2·98	<1·60	7·96	7·69	7·98	6·80
	44	<1·60	<1·60	8·13	7·80	7·88	7·47
5	37	3·19	<1·60	7·35	<1·60	7·33	5·76
	44	1·60	<1·60	7·05	<1·60	7·18	5·72
6	37	2·87	<1·60	7·93	5·09	8·26	<1·60
	44	<1·60	<1·60	7·22	<1·60	7·99	<1·60
7	37	3·15	<1·60	7·20	5·78	8·34	6·84
	44	<1·60	<1·60	7·00	5·72	8·25	<1·60
8	37	3·25	<1·60	8·16	7·78	8·38	<1·60
	44	2·08	<1·60	8·12	7·54	8·21	<1·60

* Mixture of 1 ml of brine and 10 ml of broth.

These observations confirmed the earlier indications that staphylococci occur frequently in ordinary bacon curing brines, and hence might be expected on bacon. The brines referred to here were matured brines used over long periods, as in traditional practice: the situation might obviously be different with short term synthetic brines.

The occurrence of staphylococci on normal and on vacuum packed bacon

Three different kinds of bacon were examined: (a) normal bacon; bought locally, sliced at the point of sale and examined at once; (b) samples of freshly made vacuum packed bacon obtained direct from the factory and examined at once; (c) samples of vacuum packed bacon bought locally. The bacon in (b) was packed with hygienic precautions but, as far as is known, without any substantial modification of the curing procedure, so it may be regarded as reasonably representing the condition of freshly cured bacon ready to leave the factory. Thus, comparison of (a) with (b) reveals the influence of the processes of distribution and sale with normal bacon whereas comparison of (b) with (c) shows the effect of the corresponding processes with vacuum packed bacon. Data from these examinations are compared in Table 5.

Table 5. *A comparison of bacterial counts on PP agar of bacon taken directly from the factory or bought in shops*

Type and source of sample		No. of samples	Log_{10} (colony count/g) at		
			20°	37°	44°
Shop samples of loose, sliced bacon from shop no.	1	1	7·4	8·1	<3·3
	2	1	7·2	7·1	6·4
	3*	1	7·8	7·1	4·7
	4	2	8·2	7·6	<3·3
			8·3	7·5†	5·7
	5	2	8·0	7·9	<3·3
			8·2	8·3	5·8
	6	1	7·3	6·8†	6·0
	7	1	7·5	7·4	5·5
Vacuum packed bacon direct from factory	A‡	3	3·7–4·5	3·5–4·5	<3·3
	A§	3	4·3	3·9–5·2	<3·3–5·0
	B	3	4·0–5·2	<3·3–4·1	<3·3
	C	3	3·9–5·2	<3·3	<3·3
Shop samples of vacuum packed bacon from factory	A	3	3·7–4·7	<3·3–3·7	<3·3
	B	3	5·0–8·0	<3·3–7·2	<3·3
	D	3	6·6–7·5	5·2–6·1	<3·3

* Based on MPN results, samples of bacon taken from shop no. 3 carried 13 and 75 coagulase positive *Staph. aureus*/g respectively.

† These were the only counts at 37° or at 44° showing phosphatase positive colonies; counts of these were 10^4/g in both cases.

‡ Green bacon. § Smoked bacon.

Normal bacon from retailer. With the sliced bacon bought locally, counts up to 8×10^4/g at 37° and $2{\cdot}5\times10^6$/g at 44° were found. These high values at elevated temperatures indicate that, between maturation in the factory (at a temperature near 5°) and the time of retail sale, the bacon had acquired large numbers of mesophilic bacteria, the sources of which are at present unknown. From two samples bought at different times from shop no. 3, counts of about 13/g and 75/g of *Staph. aureus* were obtained using the MPN technique. In view of the small number of samples studied the occurrence of coagulase positive staphylococci in two of them suggests quite frequent contamination.

Vacuum packed bacon from manufacturer. On the freshly cured bacon the largest part of the population was made up of micrococci (growing at 20° but not at 37° or 44°) which are normally present during the cure. Bacteria able to grow at 44° (e.g. staphylococci) were found in only 3 of the 18 packs examined and bacteria able to grow at 37° in only 7 of them. No coagulase positive organisms were found.

Vacuum packed bacon from retailer. An appreciable proportion (5/9) of packs of bacon bought in the shops gave 37° counts higher than 10^5/g, but organisms growing at 44° were not detected at the level of 2×10^3/g. However, during storage experiments with commercial sealed packs, one was found which contained *Staph. aureus* of a phage type associated with food poisoning at a level of 3×10^6/g. In comparison with the counts revealed above, this number seemed too high to be the result of chance contamination and thus suggested that the organisms had grown in the pack.

These observations show that potentially dangerous staphylococci can occur on normal bacon, though it appears that they are most likely to be added after curing. (This question, not being immediately relevant to the problem with packed bacon, was set aside for the present.) None was found in the small number of fresh packs of bacon examined, but they can clearly occur there, for they were found in one pack stored unopened in sufficient numbers to suggest that multiplication had occurred.

Growth of Staph. aureus *on bacon packed in the absence of oxygen*

After the above experience, it seemed necessary to confirm that *Staph. aureus* could grow on bacon packed anaerobically. The bacon was packed in cans under nitrogen to provide defined anaerobic conditions. At the same time, because known numbers of food poisoning *Staph. aureus* were to be inoculated, the opportunity was taken to repeat a comparison of the several possible culture media and incubation temperatures. The results (Table 6) show that at 37° the 110 agar count and the mannitol positive count on MS agar both included a preponderance (particularly large with the green bacon) of organisms which were not phosphatase positive. This confirms the choice of the PP medium.

Table 6. *Bacterial counts of smoked and unsmoked bacon inoculated with* Staphylococcus aureus *and stored at anaerobically* 20°

Type of bacon	Days of storage	Log$_{10}$ (colony count/ml)* at									
		37°, on media†					44°, on media†				
		PP, of		MS, of		110, of	PP, of		MS, of		110, of
		(a)‡	(b)‡	(a)‡	(c)‡	(a)‡	(a)‡	(b)‡	(a)‡	(c)‡	(a)‡
Unsmoked	0	3·40	2·70	3·51	3·03	3·40	2·67	2·32	2·49	2·37	2·21
	3	6·44	4·02	6·62	6·62	6·60	4·67	3·74	4·55	4·27	3·80
	6	6·27	4·24	6·45	6·43	6·46	3·89	3·89	3·85	3·84	3·67
	13	6·28	5·09	6·54	6·40	6·46	4·14	3·83	4·48	4·10	4·29
Smoked	0	3·87	2·54	3·83	3·44	3·56	2·60	2·51	2·58	2·42	2·28
	3	4·57	3·23	4·86	4·70	4·61	3·68	3·30	4·30	3·91	3·74
	6	3·94	3·16	4·07	4·00	3·88	3·06	3·06	3·21	3·08	2·73
	13	4·74	4·32	4·99	4·89	4·89	3·44	3·26	3·41	3·56	3·26

* Mean counts from three cans.

† Media: PP, phenolphthalein phosphate agar; MS, mannitol-salt agar; 110, staphylococcus medium 110.

‡ (a), total colonies; (b), phosphatase positive colonies; (c), mannitol positive colonies.

The PP counts at 44° corresponded well with the numbers of *Staph. aureus* inoculated and, following these counts through the period of storage (Table 6), it is evident that multiplication of the introduced staphylococci must have been very limited. The same general conclusion may be drawn from the parallel counts on the MS and 110 agars.

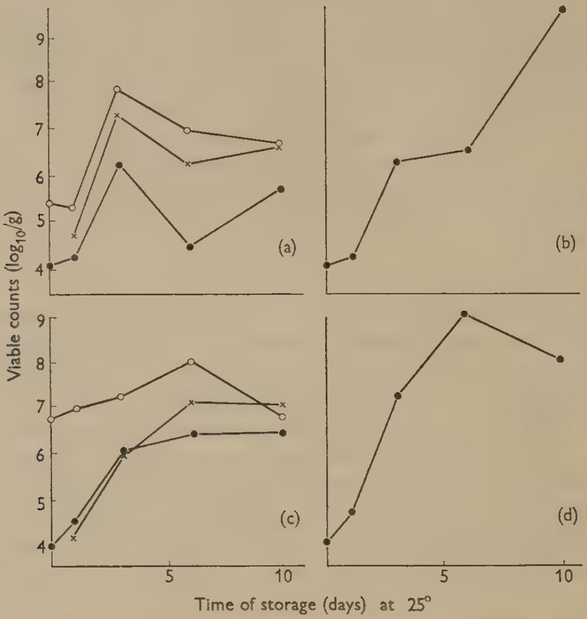

Fig. 1. The effect of total suppression, by irradiation, of the normal flora of smoked (a and b) and unsmoked (c and d) bacon on the growth of *Staphylococcus aureus* introduced just before storage at 25° in packs containing nitrogen. Unirradiated, (a) and (c); irraditaed with 5 Mr, (b) and (d).
Crosses, lactobacilli; full circles, *Staph. aureus*; open circles, micrococci; continuous lines, unirradiated bacon; dashes, bacon irradiated with 5 Mrad.

Two possible reasons were suggested for this failure of the introduced staphylococci to develop: either the bacon was unsuitable as a substrate, or the presence of other bacteria on the bacon caused an inhibition. To distinguish these possibilities, inoculations were made into packs in which the bacteria on the bacon had first been killed, and into parallel untreated controls. Attempts to sterilize the bacon with ethylene oxide were unsatisfactory as the sterilized bacon remained generally inhibitory. The bacon was therefore sterilized by irradiation.

Unsmoked and smoked bacon samples from the same factory were sterilized by irradiation (10 Mrads), inoculated with a food poisoning strain (58/7779) at a level of about 10^4/g and stored at 25°. After incubation for 1, 2 and 5 days different cans were taken as samples and dilutions of homogenates of the contents were plated on PP agar and incubated at 44°. In 5 days there was roughly a thousandfold increase in this count (10^4–10^7/g), there being little difference between the samples of green and smoked bacon. The experiment was then repeated in a modified form. Unsmoked and smoked bacon samples from another factory were inoculated as above both with and without prior sterilization with 5 Mrads. Besides the counts made on PP agar at 44° for *Staph. aureus*, counts were also made on PP agar at 20° and on Rogosa agar (Oxoid) incubated for 4 days in 10% CO_2 in H_2. The counts of tolidine (or benzidine; Deibel & Evans, 1960) positive colonies at 20° were taken to represent micrococci and the Rogosa agar counts to represent lactobacilli. The results (Fig. 1) were similar to those of the first experiment except that the staphylococci grew more slowly on the smoked bacon. A similar experiment was carried out in which the bacon (from a third factory) received only a 'pasteurizing' dose of 1 Mrad, so that its indigenous flora was only reduced in numbers, not eliminated. The results are given in Fig. 2; again there was less growth of staphylococci where the normal flora was not reduced, and especially on the smoked bacon.

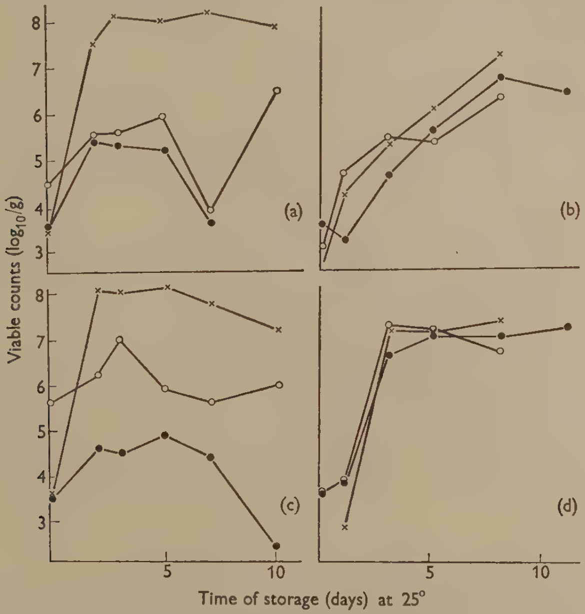

Fig. 2. The effect of partial suppression, by irradiation, of the normal flora of smoked (a and b) and unsmoked (c and d) bacon on the growth of *Staphylococcus aureus* introduced just before storage at 25° in packs containing nitrogen. Unirradiated, (a) and (c); irradiated 1 Mr, (b) and (d).
Crosses, lactobacilli; full circles, *Staph. aureus*; open circles, micrococci; continuous lines, unirradiated bacon; dashes, bacon irradiated with 1 Mrad.
The bacon was different from that used in the experiment illustrated by Fig. 1.

An interesting incidental inference from these experiments is that even severe irradiation treatments did not produce inhibitory substances in the bacon, nor was there any detectable destruction of the nitrite to complicate the issue.

The important conclusions to be drawn, however, are that *Staph. aureus* could grow well on this bacon stored anaerobically at 25°, but did so more rapidly and attained greater numbers when the bacon had been partially or wholly sterilized by irradiation. It seems that under the conditions of these experiments, and with the inoculum size used, *Staph. aureus* did not compete successfully with the natural flora of the bacon. Further, the inhibition might be due to the lactobacilli in the bacon flora, for the main difference between the treated and control samples was in the numbers of lactobacilli present during the period when the staphylococci were multiplying (Fig. 2).

Discussion

These observations suggest that coagulase positive staphylococci occur in a high proportion of bacon brines, though only in numbers between 1 and 50/ml, and that they can survive there almost indefinitely. There is therefore every reason to expect them to occur on bacon, in small numbers—a few were indeed found on 2 out of only 9 samples of normal bacon examined. Though these observations are limited, they suffice to suggest that normal bacon must be regularly contaminated with potentially food poisoning staphylococci and, therefore, that the occurrence and behaviour of these pathogens in vacuum packed bacon needs consideration.

It is interesting that, to judge from the few samples we have compared, vacuum packed bacon is 'cleaner' than normal bacon if the two are compared at the point of retail. This is especially so in respect of bacteria capable of growth at 44°, which perhaps reflect miscellaneous contamination acquired by normal bacon during slicing and retail; more information about this aspect seems desirable.

Nevertheless, even among the few samples of commercial vacuum packed bacon examined, one has already been encountered which, after storage at 25°, contained staphylococci of a phage type associated with food poisoning, in such numbers as to suggest that growth had probably taken place. Our experiments with inoculated packs have not confirmed this possibility, but the experiments of Thatcher *et al.* (1962) suggest that, starting roughly from conditions similar to those observed in this particular pack, it is possible for growth to continue to the point of toxin production.

This chain of evidence, though slender as yet, suggests that there is no guarantee against the possibility of toxicity if packed bacon is exposed to warm conditions.

It seems remarkable that bacon has never been the cause of staphylococcal food poisoning. The problem is of considerable general interest microbiologically for, according to present observations, it seems that the principal obstacle to development of the staphylococci (which, we now conclude, probably occur frequently on bacon) is the usual preponderance of the normal bacterial flora. It would be interesting to know why this flora inhibits the staphylococci. It is clear from our earlier experience (Ingram, 1960), from that of Cavett (1962) and from the present experiments, that this inhibition is likely to fail if the bacon is stored at temperatures exceeding about 25°.

If the view (Hodge, 1960) is correct, that it is impossible to exclude coagulase positive staphylococci from handled food, and from packed bacon in particular, then it is evidently desirable for safety that it should be kept at a temperature at which these organisms do not grow readily. The experience of others (Haines, 1934; Evans & Niven, 1950), which we have crudely confirmed with 9 of the strains used in these experiments, is that the growth of these organisms is rapid at temperatures above 20° and negligible below 10°. At least it should be required that packed bacon carry the instruction 'keep in a cool place'. Temperatures below 10° would give complete protection. It may prove premature that uncooked bacon has recenty been specifically excluded from the scope of Food Hygiene Regulation no. 25 (Regulation, 1960).

REFERENCES

BARBER, M. & KUPER, S. W. A. (1951). Identification of *Staphylococcus pyogenes* by the phosphatase reaction. *J. Path. Bact.* **63**, 65.

BUTTIAUX, R. & MORIAMEZ, J. (1958). Le comportement des germes tests de contamination fécale dans les saumures de viandes. *Proc. 2nd Int. Symp. Fd Microbiol.*, p. 247. London: H.M.S.O.

CAVETT, J. J. (1962). The microbiology of vacuum packed sliced bacon. *J. appl. Bact.* **25**, 282.

CHAPMAN, G. H. (1945). The significance of sodium chloride in studies of staphylococci. *J. Bact.* **50**, 201.

CHAPMAN, G. H. (1946). A single culture medium for selective isolation of plasma-coagulating staphylococci and for improved testing of chromogenesis, plasma coagulation, mannitol fermentation, and the Stone reaction. *J. Bact.* **51**, 409.

CHAPMAN, G. H. (1948). An improved Stone medium for the isolation and testing of food-poisoning staphylococci. *Food Res.* **13**, 100.

DEIBEL, R. H. & EVANS, J. B. (1960). Modified benzidine test for the detection of cytochrome-containing respiratory systems in micro-organisms. *J. Bact.* **79**, 356.

EVANS, J. B. & NIVEN, C. F. (Jr.) (1950). A comparative study of known food-poisoning staphylococci and related varieties. *J. Bact.* **59**, 545.

HAINES, R. B. (1934). The minimum temperatures of growth of some bacteria. *J. Hyg., Camb.*, **34**, 277.

HODGE, B. E. (1960). Control of staphylococcal food poisoning. *Publ. Hlth Rep., Wash.*, **75**, 355.

INGRAM, M. (1960). Bacterial multiplication in packed Wiltshire bacon. *J. appl. Bact.* **23**, 206.

INGRAM, M. (1962). Microbiological principles in prepackaging meats. *J. appl. Bact.* **25**, 259.

INGRAM, M. & HOBBS, B. C. (1954). The bacteriology of 'pasteurized' canned hams. *J.R. sanit. Inst.* **74**, 1151.

KITCHELL, A. G. (1958). The micrococci of pork and bacon and of bacon brines. *Proc. 2nd Int. Symp. Fd Microbiol.*, p. 191. London: H.M.S.O.

KOCH, F. E. (1942). Electivnährboden für Staphylokokken. *Zbl. Bakt.* (Abt. 1 Orig.) **149**, 122.

REGULATION (1960). The Food Hygiene (General) Regulations, no. 25, 1960, p. 10. London: H.M.S.O.

RICHARDS, T., SOULIDES, D. A. & SOULIDES, E. (1945). The inhibitory effect of crystal violet, sodium azide and thallous acetate upon mastitis streptococci and other common milk flora. *Prod. Soc. appl. Bact.* p. 44.

SEVEL, B. & PLOMMET, M. (1960). Adaptation d'un milieu sélectif à l'isolement des staphylocoques de mammite. Application au diagnostic d'étable. *Lait* **40**, 2.

SHAW, C., STITT, J. M. & COWAN, S. T. (1951). Staphylococci and their classification. *J. gen. Microbiol.* **5**, 1010.

THATCHER, F. S., ROBINSON, J. & ERDMAN, I. (1962). The 'vacuum pack' method of packaging foods in relation to the formation of the botulinum and staphylococcal toxins. *J. appl. Bact.* **25**, 120.

ZEBOWITZ, E., EVANS, J. B. & NIVEN, C. F. (Jr.) (1955). Tellurite-glycine agar: a selective plating medium for the quantitative detection of coagulase-positive staphylococci. *J. Bact.* **70**, 686.

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(Symposium on The Effect of the Newer Forms of Packaging on the Microbiology and Storage Life of Various Foods: Paper I)

SOME PROPERTIES OF FOOD PACKAGING MATERIALS WHICH RELATE TO THE MICROBIAL FLORA OF THE CONTENTS

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1. Introduction

The problem of selecting a packaging material for use with a foodstuff is complicated both by the range of properties of the food which must be taken into account and by the wide variety of packaging materials which can be considered. Packaging materials range from highly impermeable opaque materials, such as aluminium foil, to highly permeable transparent materials, such as cellulose acetate. The foodstuffs range from dried processed products to living vegetable tissue.

Compatibility between pack and product is the basic requirement and can be considered from many points of view. Most of these can be ignored in the present discussion, which is concerned specifically with the microbial condition of the contents of the package; but it must be emphasized that all aspects must eventually be taken into account if the pack is to enjoy successful practical use. For example, the effect of the product on the package may be as important as the effect of the package on the product, a typical case being the need for a grease resistant package for oily or greasy products.

A range of other requirements must also be noted. These relate to the suitability of the package for production and marketing. A material which is highly successful for functional reasons may be rejected in the final assessment if, for example, it is difficult to handle in the factory, does not print well, is too expensive, is not strong enough to withstand market conditions or is so strong that it cannot be opened without great difficulty. The effect of some factors of this nature is sufficiently strong to exert an indirect effect on the performance of the final pack by restricting the choice of materials which can be used. Cost is a typical example. A requirement for transparency, which is commonly encountered with food products, also restricts the choice of materials and furthermore exposes the contents to light which, in some modern shop conditions, can be quite intense.

Properties which are important from these wider points of view will not, in general, be considered in the following discussion. The treatment has also been simplified by considering only packaging materials which are available or potentially available in commercial quantities.

Broader information in the field covered by this paper can be found in a number of references of general character, e.g. Encyclopaedia (1961, 1962*a*, 1962*b*), Barail (1954), Oswin (1954) and Paine (1957), and bibliographies such as those of Baker & Foskett (1958) and Larsen (1959).

2. TYPES OF PACKAGING MATERIAL

Most of the important principles are best illustrated by concentrating on the use of flexible materials. These are of two main types, single component and multicomponent.

(a) *Single component materials*

The simpler materials are homogeneous films made either from a single chemical substance, usually polymeric, or from an intimate mixture of two or more substances. These, together with comments on their manufacture, are listed in Table 1.

Paper in various forms, including imitation parchment and glassines, is the oldest of the materials in common use. Closely related are the regenerated cellulose films which have now been available for over 50 years. The raw cellulosic material is plasticized with glycerol or similar substances and the resulting film is usually known as the PT (i.e. plain, transparent) grade.

The next material in large scale use is polyethylene, a polyolefine usually referred to in Britain as polythene. Various methods of polymerizing the ethylene are now used and the materials available range from the well established low density material (density approximately 0·92) to high density materials (density approximately 0·96) with less flexibility but with lower permeability and higher softening temperature (Raff & Allison, 1956; Renfrew & Morgan, 1960).

The newest material in the polyolefine family is polypropylene which is similar in many respects to a high density polyethylene.

Polyvinyl chloride (PVC) is an older film which has been used extensively for general manufacturing purposes but only to a limited extent for food packaging owing to earlier difficulty in obtaining plasticizers suitable for use with foods. Such plasticizers are now available.

Table 1. *A list of the commoner single component packaging materials with their typical methods of manufacture*

Material	Typical method of manufacture
Paper	
Regenerated cellulose (PT grade*)	From wood pulp treated with carbon disulphide and acid: plasticized with polyalcohols
Low density polyethylene	Ethylene polymerized at high pressure and high temperature
High density polyethylene	Ethylene usually polymerized by catalytic process at lower pressures and temperatures
Polypropylene	Catalytic polymerization of propylene: frequently biaxially orientated by stretching
Polyvinyl chloride	Varying degrees of plasticizing are common
Polyvinylidene chloride co-polymers	Vinylidene chloride co-polymerized with vinyl chloride or other monomers: frequently biaxially orientated
Rubber hydrochloride	From rubber and hydrogen chloride: varying degrees of plasticising are common
Cellulose acetate	A plasticized product usually made by solvent casting
Polystyrene	Biaxial orientation often used to increase toughness
Polyester	Condensation of ethylene glycol and terephthalic acid
Polyamide { nylon 6	Polymerization of cyclohexanone
Polyamide { nylon 11	Polymerization of amino-undecanoic acid derived from castor oil

* PT, plain, transparent.

Polyvinylidene chloride (PVDC), which must not be confused with PVC, is without doubt one of the outstanding of the present generation of films, the permeability of even very thin films being extremely low. It is finding increasing usage, usually in the form of a co-polymer with, for example, vinyl chloride.

Of the remaining materials in Table 1 only the polyamides or nylons call for specific mention in that there are considerable differences between the chemical structure of the various members of the family, the only general similarity being that the polymerizing linkage is an amide bond.

This list of materials is in itself extensive: but there is added complexity in that the detailed properties of any one material depend to a considerable extent on the precise chemical formulation, including the length of the polymer chains and the presence of minor additives. It also depends on the method of manufacture; for example, orientation of the film during extrusion affects the strength, clarity and permeability. The inexperienced user should therefore exercise the greatest care before assuming that a given sample of material has known reproducible properties which can be characterized by giving it a simple generic name.

(b) *Multicomponent materials*

Extensive as the list in Table 1 may be, it is in practice insufficient to meet many demanding requirements and a range of multilayer materials has been developed to overcome the limitations of the single component films. A few typical examples are given in Table 2.

Table 2. *Some typical multicomponent packaging materials and their method of manufacture*

Material		Typical method of manufacture
Paper coated with wax		Wax applied as hot melt
Paper/polyvinylidene chloride co-polymer*		Solvent or emulsion coating
Cellulose, grade†	MSAT	Two sided solvent coating with mixture of nitrocellulose, wax, etc.
	QSAT	Similar to MSAT but coating contains less wax
	MSADT	One sided nitrocellulose coating
	MXXT	Two sided coating with polyvinylidene chloride co-polymer
	MXDT	One sided polyvinylidene chloride co-polymer coating
Nitrocellulose/cellulose/polyethylene*		MSADT cellulose with polyethylene extruded on cellulose surface
Polyvinylidene chloride co-polymer/cellulose/polyethylene*		MXDT cellulose with polyethylene extruded on cellulose surface
Polyester/polyethylene*		Extrusion coating of polyethylene or adhesion bonding of two preformed films
Paper/foil/polyethylene*		Foil adhesion bonded to paper and extrusion coated with polyethylene

* The symbol / denotes a coating or bonding between the two surfaces: the material quoted first is usually used on the outside of the package.

† Method of designation used by many, but not all, manufacturers: M, moisture proof; Q, semi-moisture proof; S, heat sealable; A, anchored coating; T, transparent; D, one-sided; X, polyvinylidene chloride co-polymer.

The simplest treatment is to apply a surface coating to one of the films in Table 1. Wax coatings, for example, have been in use for many years with paper and board to give moderately protective barriers. They also give the material very useful heat sealing properties. Coatings are also commonly applied to cellulose films. These are usually based on a mixture of nitrocellulose and a wax, the composition being adjusted to give materials with two types of final performance, namely MSAT grades which are relatively impermeable and QSAT grades which are moderately permeable. The most recent development has been to use coatings of vinylidene chloride co-polymer, the resulting combination (MXXT grade) having very low permeability.

In some instances the coating may be applied to one side only of the cellulose film. Such nitrocellulose lacquered material (MSADT grade) has a special use for meat packaging, but also forms the basis for combination with polyethylene. The latter is extrusion coated on to the uncoated surface of the cellulose to form a triple layer laminate which is used extensively for packaging of bacon. A similar laminate based on cellulose coated on one side only with vinylidene chloride co-polymer (MXDT grade) is also coming increasingly into use.

The many possible combinations of material cannot be discussed at length. It is sufficient to note that virtual impermeability can only be achieved at present by incorporating aluminium foil in the laminate; four-component materials of this type are in common use for packaging dehydrated foods. Such materials are essentially not transparent.

3. THE PERMEABILITY OF PACKAGING MATERIALS

The most important property from the microbiological point of view is the permeability to gases, particularly water vapour and oxygen.

At first sight it would appear that copious information regarding permeabilities is available in the literature and that the performance of any given package could be predicted to a reasonable degree of accuracy using only published data. In practice, however, this is usually possible to a very limited extent only. Some important figures do not appear in the literature, and the values of others show considerable lack of consistency in the reports of different authors. It is often correspondingly difficult to decide which permeability value should be used in calculating the performance of the package.

Table 3. *Typical permeabilities* to water vapour and oxygen of packaging materials*

Material		Permeability to	
		Water vapour†	Oxygen‡
Cellulose, grade§	PT	500	<1 when dry 3 at 50% R.H. 5,000 at 100% R.H.
	QSAT	150–350	2,000 at 100% R.H.
	MSAT	3	1·5 at 50% R.H.
	MXXT	1·5	<1 at 50% R.H.
Low density polyethylene		5	3,000–10,000
High density polyethylene		1·5	500–3,000
Polypropylene		1·5	1,500
Polyvinyl chloride**		10	200
Polyvinylidene chloride co-polymer		0·5	3
Rubber hydrochloride**		2–80	100–5,000
Cellulose acetate**		500	—
Polystyrene		50	4,000
Polyester		10	50
Nylon	6	15	25
	11	5	300

* These figures, which refer to materials approximately 0·001 in. thick, are based partly on data given in the literature and partly on practical observations on a limited number of samples: they indicate only the probable order of magnitude and not the detailed performance which can be expected from materials from different sources of supply.

† Results given as $g/m^2/24$ hr at 25° and 75% relative humidity (R.H.).

‡ Results given as $ml/m^2/24$ hr at 20°. § The grades are specified in the footnotes to Tables 1 and 2.

** The precise permeability is markedly dependent on the plasticizer concentration.

This situation arises from a number of causes. First, materials with the same generic name may, as already mentioned, differ in properties due to differences in detailed formulation and manufacturing technique. Second, even with the products of one manufacturer there is an inherent variability of production; for example, it is difficult to maintain precise control of the thickness of a plastic extruded film only 0·001 in. thick. Third, environmental factors can sometimes affect the permeability; this is particularly so in the case of cellulose films which are greatly affected by humidity; protective coatings can be disrupted by even moderate mechanical damage such as folding. Fourth, test methods and their interpretation vary widely; there is a great need for convenient, reproducible methods of proven relevance to packaging problems. The total effect of these factors is such that precise calculations should, in the view of the writer, usually be based only on experimentally determined permeabilities of the material under test and all theoretical predictions should be confirmed by a practical test with finished packages under conditions as near as possible to those of actual use.

With this limitation in mind the figures in Table 3 are shown as an indication of the orders of magnitude which can be expected.

Stannett *et al.* (1962) presented a useful survey of the influence of various factors on permeability and gave references to earlier work, and Charlton & DeLong (1956) reviewed the subject from the point of view of food packaging.

(a) *Permeability to water vapour*

Permeability to water vapour is often expressed in Britain in terms of grammes of water vapour passing through a square metre of the material in 24 hr at known temperature, with known humidity on one side and zero humidity on the other. A thickness of 0·001 in. is usually specified.

It can be seen from Table 3 that there are broadly three types of material. First, those with high permeability (of the order of hundreds of $g/m^2/24$ hr/0·001 in. thickness) under typical warm room conditions, e.g. cellulose acetate, polystyrene and cellulose of the PT and QSAT grades. Second, those with medium permeability, approximately 10–50 $g/m^2/24$ hr/0·001 in. thickness; this group includes most of the commoner organic polymers. Third, those with low permeability, of the order of a few $g/m^2/24$ hr/0·001 in. thickness; vinylidene chloride co-polymers are the least permeable of the transparent films and these are followed by high density polyethylene and polypropylene.

(b) *Permeability to oxygen*

Relative permeabilities to oxygen and water vapour are often similar, polyvinylidene chloride co-polymers having low permeability to both. This, however, does not always apply, polyethylene being an important example with low permeability to water vapour but high permeability to oxygen.

The values for oxygen permeability given in Table 3 are for ml of gas transmitted through a square metre of material in 24 hr under a pressure differential of 1 atmosphere of the test gas. This unit is in less widespread use but is convenient for simple calculations with normal packaging problems.

Particular note should be taken of the cellulose materials. The completely dry material is highly impermeable whereas wet material is thousands of times more permeable. The main purpose of the coatings in MSAT grades is to keep the cellulose dry and therefore less sensitive to the effects of high humidity. The polyvinylidene chloride co-polymer coating used in MXXT films not only keeps the cellulose dry but also adds its own contribution to the barrier properties.

The materials in current use which are most impermeable to oxygen are based on polyvinylidene chloride co-polymer, dry cellulose or aluminium foil and they are frequently combined with polyethylene to increase the mechanical strength, to give reliable heat sealing and to increase the resistance to water and water vapour. This is the basis for the cellulose-polyethylene combinations used for bacon pouches.

Low permeability, however, is not always required. In the packaging of fresh meat an oxygen permeable material is required to keep the meat pigments in an oxygenated form. One approach is to use MSADT cellulose film with the uncoated cellulose surface in contact with the meat. The cellulose is thus wetted and transmits oxygen, while the coating on the outer surface prevents undue loss of water vapour and consequent desiccation of the meat. Thin films of polyethylene highly permeable to oxygen and relatively impermeable to water vapour can also be used in a similar manner.

(c) *Permeability to carbon dioxide*

Permeability to carbon dioxide is known to be a most important property with a few products which evolve this gas, but its precise significance with true respiring systems has been less investigated. As a broad generalization the permeability of most films to carbon dioxide usually parallels that to oxygen and is several times greater. The ratio appears to be greatest with hydrophilic materials such as wet cellulose where it can be 20 or more, and is least with hydrophobic materials, where it may be only 2 or 3. In practice this means that most common materials, with the exception of polyvinylidene chloride co-polymer, are appreciably permeable to carbon dioxide.

(d) *Permeability to other gases*

Permeability to organic vapours is frequently important in general packaging investigations. With food products this mainly concerns the ability to contain odorous substances, a subject which has little direct microbiological significance.

Permeability to sulphur dioxide is relevant, however, in view of its use as a food preservative. Few quantitative figures are available but as a working assumption it seems reasonable to regard permeability to sulphur dioxide as paralleling that to carbon dioxide and oxygen.

(e) *The effect of temperature*

Permeation of the commoner gases appears to follow normal physical laws fairly well in its dependence on temperature. the logarithm of the permeability increasing proportionately with the absolute temperature. As a broad generalization the permeability for most combinations of gas and film which are of present interest increases

1·5–3-fold for every 10° rise in temperature, but the literature contains many examples of ratios outside this range. The writer has not attempted to review the quantitative significance of these figures but it is clear that from a qualitative point of view the temperature coefficient of permeability is considerably less than that of biological activity, particularly respiration. It is therefore at least theoretically possible for a packaged respiring product to have an adequate supply of oxygen at low temperatures and become anaerobic at higher temperatures. Further study of this type of relationship at refrigerator and room temperatures is highly desirable. In the former case artifacts due to condensation of water must be taken into account.

(f) *The effect of film thickness*

The effect of thickness cannot be discussed here in detail. It is sufficient to note that with thin homogeneous films the permeability is more or less inversely proportional to thickness. Above about 0·01 in., however, the effect of increased thickness is less marked.

(g) *The permeability of complete packs*

The discussion so far has been limited mainly to the properties of sheets of packaging material. These can be used to enclose a product in a variety of ways.

The easiest way is by a simple wrapping operation, but it must be recognized that this will inevitably leave gaps in the protection. The relative importance of such gaps will depend on the permeability of the film, being greatest with the highly impermeable materials.

For such materials it is usual to arrange for heat sealed joins, the most efficient seals being obtained with flat pouches. Great care is needed where there is a danger of creases running through the seal, but even if this is avoided the permeability in the region of the seal may well be higher than in the body of the pack. Again there are few quantitative figures, particularly of the performance of badly made (e.g. overheated) seals.

Some materials cannot be heat sealed and have to be closed by mechanical means such as clips. These are frequently only moderately efficient and tend to be variable in performance.

The practical approach to testing the efficiency of seals is usually to carry out direct tests on the finished pack under representative test conditions, preferably including cycling of temperature and humidity. The influence of bulk packaging of retail units into outer cases and the stacking of such cases in warehouses can also be studied in this way.

4. THE EFFECT OF WATER CONDENSATION

An effect which is related to permeability in complete packs is the formation of internal condensate in air pockets where the film lies loosely over moist surfaces. The amount of condensation depends on the relationship between external and internal temperatures and on the internal humidity; it also depends on the water vapour permeability and the water absorption of the film. Accumulations of free water, with local dilution effects on the surface of the product, provide excellent focal points for surface spoilage and condensation is one of the factors which limit the shelf life of many packs.

5. Vacuum Packs

It is common practice with many products to evacuate the pack before sealing, either by inserting a probe into the neck of the pack or by enclosing the pack in a chamber which is then evacuated. This results in the familiar tightly drawn packs known as vacuum packs.

There is a general impression in some circles that such packs contain a high vacuum of over 20 in. of mercury, since such a reading was registered by the evacuating equipment at the moment of sealing. It will be readily apparent however, that this cannot occur with a truly flexible material which will collapse under external pressure, unless there are considerable voids in the product into which the material is prevented from collapsing. Such voids occur in dehydrated foods, for example, or with eviscerated poultry, but to a much smaller extent with products such as bacon. The physical vacuum inside an efficiently evacuated bacon pouch therefore has little significance other than to indicate the voids in the pack and the inflexibility of the material. It would seem that the quantity of air left inside and the intimate physical contact between product and film are the important considerations.

6. Three Dimensional Packs

The problem of obtaining an efficient and attractive package of bulky articles has already been mentioned. A folded wrap can be used, but this can usually not be sealed completely. The alternative is a fully sealed flat pouch, but this is seldom attractive in appearance.

The simplest variation is to make the pouch of a material with high elasticity or ability to shrink on warming. The resulting stretch wraps or shrink wraps conform closely to the contours of the packaged product and present an attractive appearance. Polyvinyl chloride, polyvinylidene chloride co-polymer, rubber hydrochloride and polyethylene are all available for this type of packaging.

The other approach is to preform the container to the desired shape. Most of the plastic materials can be used to form trays, boxes and bottles all of which can be sealed hermetically if required. These will not be discussed in detail except to note that the walls of such semirigid containers tend to be thicker than the flexible films and the permeability correspondingly lower. On the other hand, laminated materials cannot be used and there are technical limitations in applying efficient coatings. Preformed plastic containers having the low permeabilities characteristic of efficient laminates or coated films are not therefore generally available.

7. Sterilization in the Package

The last properties for discussion are those which lend themselves to sterilization in the package.

The most direct method is by the application of heat (Davis, Karel & Proctor, 1959, 1960; Keller, 1959; Land, 1962; Wornick, Karel & Proctor, 1960). High density polyethylene, polypropylene and nylon are typical modern heat sealable materials which will withstand retorting temperatures. Polyester withstands these conditions but is not directly heat sealable. Sterilization of foods in such packs by conventional retorting techniques is therefore quite feasible. Care is needed in practical application, however, since the containers are easily ruptured or weakened by pressure changes in the retort, and the reliability of the heat seals and the resistance of the material to puncture during storage need to be considerably greater than in more conventional packaging applications. Probably a greater limitation, however, is that the lowest orders of oxygen permeability cannot be achieved with present sterilizable materials, unless aluminium foil-containing pouches are acceptable; most heat treated products are sensitive to oxidation and are expected to withstand long storage. We are still, therefore, some way from a plastic container which will be truly competitive with the metal can.

An alternative method of sterilization in pack merits a mention; this is by treatment with such gases as ethylene oxide or propylene oxide to which films such as polyethylene are fairly permeable. This method is commonly used with medical products, but application to foodstuffs is complicated by questions of the medical acceptability of the toxic gases.

8. General Comments

This discussion has necessarily been restricted in its scope but should serve to outline many of the properties of packaging materials which are important from the microbiological point of view. Their influence on the product can usually only be incompletely predicted on the basis of present evidence and there is, in general, no substitute for practical packaging tests under the proposed conditions of use. Such tests should be performed whenever possible. They will also permit an appreciation of the many other factors which do not affect the microbiological condition of the product but which must be taken into account before a package of practical utility can be developed.

9. References

Baker, E. A. & Foskett, D. J. (1958). *Bibliography of Food.* London: Butterworth.

Barail, L. C. (1954). *Package Engineering.* New York: Reinhold.

Charlton, F. S. & DeLong, R. F. (1956). The role of permeability in food packaging. *Paper, Film & Foil Converter* **30** (4), 19; **30** (5), 22.

Davis, E. G., Karel, M. & Proctor, B. E. (1959). Testing the bursting strength of plastic bags for processed foods. *Mod. Packag.* **33** (4), 135.

Davis, E. G., Karel, M. & Proctor, B. E. (1960). Heat processing versus permeability. *Mod. Packag.* **33** (7), 208.

Encyclopaedia (1961). *Packaging Review Data Book.* London: Harlequin Press.

Encyclopaedia (1962*a*). *Modern Packaging Encyclopaedia.* New York: Packaging Catalogue Corporation.

Encyclopaedia (1962*b*). *Modern Plastics Encyclopaedia.* New York: Plastics Catalogue Corporation.

Keller, R. G. (1959). Flexible packages for processed foods. *Mod. Packag.* **33** (1), 145.

Land, W. (1962). Plastic materials to withstand sterilization processes. *Packaging* **33** (3), 66.

LARSEN, S. A. (1959). *Packaging Research: An Inventory*. New York: American Management Association.

OSWIN, C. R. (1954). *Protective Wrappings*. London: Cam Publications.

PAINE, F. A. (1957). Some aspects of packaging. *Chem. & Ind.* (*Rev.*) **52**, 1656.

RAFF, R. A. V. & ALLISON, J. B. (1956). *Polyethylene*. London: Interscience.

RENFREW, A. & MORGAN, P. (Editors) (1960). *Polythene*. London: Iliffe & Sons.

STANNETT, V., SZWARC, M., BHARGAVA, R. L., MEYER, J. A., MYERS, A. W. & ROGERS, C. E. (1962). Permeability of plastic films and coated paper to gases and vapours. *Monogr. Ser. Tech. Ass. Pulp Pap. Ind., N.Y.* no. 23.

WORNICK, R. C., KAREL, M. & PROCTOR, B. E. (1960). Heat penetration into plastics packages for heat processed foods. *Package Engng* **5** (7), 33.

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MICROBIOLOGICAL PRINCIPLES IN PREPACKING MEATS

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1. Introduction

The term prepacking covers diverse procedures, two extremes being distinguishable. First, in self-service stores, it is common to wrap meat on the premises shortly before sale; and although it may be wrapped in transparent film the wrap is seldom sealed completely. At the other extreme is a centralized packing station delivering sealed packs over a wide area, the time before consumption being several days or longer. The first type of operation differs mainly in style from the old-fashioned wrapping in paper on the counter, and offers the minimum of new microbiological problems. The second type allows time for the new type of packing to impress its influence on the microbiological situation: hence it is mainly the latter which is to be the subject of this paper. Of course there are all gradations between the two extremes indicated.

The methods used for prepacking meats are at present based on considerations quite other than microbiological. Apart from questions of convenience in use and suitability for machine handling, the packaging materials are sold on the basis that they improve the quality of the foodstuffs packed. Quality means appearance in this context, for the purchaser of the pack can neither smell nor feel the contents. Further, with meats important factors even in appearance, like 'surface bloom', cannot readily be assessed from outside the package, so that the crucial factor is the preservation of an attractive colour. Thus, current techniques for packing fresh red meats are dominated by the desire to retain the bright colour of oxygenated myoglobin, which needs air; and similarly, the main aim in packing cured meats is to prevent the colour fading, which in this case is caused by exposure to air.

Consequently, broadly speaking, fresh meats are packed in oxygen-permeable film and not subjected to a vacuum, whereas the reverse is the rule for cured meats. Certain meats can be packed by either method. White meats such as chicken do not change much in appearance with change in oxygen content of the atmosphere. Frozen meats look unattractive and to hide this they are often packed in opaque boxes which need not be sealed.

It is plain that the oxygen relations in a pack will have an important influence on its microbiology, but these relations are far from simple. Both fresh and cured meats consume oxygen at a substantial rate; further, if the meat carries many micro-organisms, as some meats do, the micro-organisms consume oxygen too, according to their nature and number. At the same time carbon dioxide, which exerts a selectively inhibitory action, is given off. It is probably the partial pressures of these gases in the internal atmosphere of the pack which influence the microbiological picture: this has been demonstrated for oxygen, but there are no critical data for carbon dioxide. These pressures in turn depend on a dynamic equilibrium between the rates of metabolism by the contents and the rate of diffusion through the wrapper, rates which vary independently with temperature.

Quantitative investigation of these complex interrelations is hampered by technical difficulties apart from microbiological problems. It is not easy with a tight pack to get a sample of the internal atmosphere adequate to establish its composition; further, before quantities can be calculated, the internal pressure and volume must be measured, and awkward unknowns like the amount of gas dissolved in the meat enter the argument. It will be some time before basic techniques and data are sufficient to describe the situation adequately. Attempts might be made to short-circuit some of the difficulties by measurements of redox potential.

A further possibly significant factor is the relative humidity inside the pack. One of the advantages of prepacking is that it diminishes the surface drying which normally detracts from the appearance of meat stored for some time. This can only mean that the humidity at the meat surface inside the pack is higher than normal, and it is well known that high humidity favours the growth of micro-organisms, bacteria especially. With this parameter, likewise, there is the problem of how to control and measure it quantitatively inside the pack, and the appropriate techniques have not yet been described.

A third factor is the bacteriological condition of the meat itself. It is commonly taken as obvious that packed meat will be less contaminated than unpacked, and simply inferred that there will be consequently less danger of spoilage or of food poisoning. This assumption overlooks the fact that meat packed in small pieces receives far more handling at an early stage of its history than is otherwise the case. Normally, on a large piece of meat micro-organisms are largely confined to the surface, whereas if it is cut so as to spread the infection over the new surfaces these organisms are given living space throughout the whole mass. In addition, if the extra processes of cutting and packing are not strictly controlled as to hygiene there is extra opportunity for unusually early contamination by the human operators, who are a likely source of food poisoning bacteria. Bearing these considerations in mind it is clear that the bacteriological advantages of prepacking meats cannot be taken for granted.

The nature of the meat itself has a profound influence on the microbiological picture. The *Pseudomonas* association of fresh meats is quite unlike the salt-tolerant *Micrococcus* association of cured meats, while the carbohydrate in (British) sausages allows an abundant production of acid, which may explain the prevalence on sausages of yeasts and lactobacilli. These differences interact with the conditions imposed by the package.

With this general background, the important microbiological factors may be considered in individual detail. Supplementary information about such factors is available in a general review (Mossel & Ingram, 1955).

2. THE EFFECTS OF TEMPERATURE

All investigators report that cool temperatures extend the storage life of prepacked meats, as would be expected from the general delay of growth and metabolism of micro-organisms at low temperature. It is further claimed (e.g. Alm, Erichsen & Molin, 1961) that any extension of storage life caused by packing is proportionately greater at lower temperatures; this may be due partly to the fact that cool conditions do not favour facultatively anaerobic species and also, perhaps, to the increased inhibitory effect of carbon dioxide (see later).

Equally important is the selective action of temperature. It has already been emphasized (Ingram, 1960) that the micrococci predominant on packed bacon at normal temperatures may be replaced by (coagulase-negative) staphylococci if it is stored at 37°. Under warm conditions the growth of clostridia within a few hours would be a danger to be expected with many meat products (Greenberg, Silliker, Nank & Schmidt, 1958; Greenberg, Silliker & Fatta, 1959). Storage below 5–10° is an effective insurance against the development of most food poisoning and similar mesophilic bacteria. It suffices merely to mention these principles, which should be well known (Ingram, 1949; Hobbs, 1953; Eddy, 1961).

The above influences of temperature seem quite straightforward, but others are less so. For instance (cf. Hannan, 1962) change of temperature alters rate of bacterial respiration more than rate of diffusion through films (i.e. the temperature coefficient is lower for diffusion than it is for metabolism): thus at lower temperatures a pack should simply on this account contain more oxygen and less carbon dioxide. Again, at low temperatures, micro-organisms became more susceptible to other restrictive influences like dryness or carbon dioxide. Such differences might be a significant but unperceived element in the overall effect of temperature on a packed product.

There is no need to enlarge here on the effects of temperature, as various examples will appear later. Selective effects on the spoilage flora are further illustrated by Cavett (1962).

3. THE EFFECT OF HUMIDITY

The influence of humidity on the growth of micro-organisms, with special reference to meat, has long been investigated in Australia by W. J. Scott and various collaborators. They established that species of *Pseudomonas* and *Achromobacter* from raw beef are inhibited by unusually small degrees of drying, growth being inhibited at equilibrium relative humidities (E.R.H.) below 95% (Scott, 1936); many of the organisms of cured meats, however, are rather resistant (limiting E.R.H. 80–90%) (Scott, 1957).

The above figures are based on observations in air, but it seems that a smaller degree of dryness might limit growth under the conditions inside a package: for example, data of Scott (1953) indicate that staphylococci can grow down to an E.R.H. of 86% under aerobic conditions, but only to an E.R.H. of 90% under anaerobic conditions. It has also been found that although growth remains possible, as the limiting degree of dryness is approached the nutritional demands of micro-organisms increase. This applies particularly to salmonellae (Christian, 1955) and staphylococci (Scott, 1953). It could conceivably be supposed, therefore, that a moderate degree of drying might provide some insurance against these undesirable bacteria, which packing would diminish. There is, however, no evidence to test this supposition.

The E.R.H. of raw meats is near to 98·5%, and that of cured meats is about 90–95% if they contain normal amounts of curing salts; these values indicate roughly the relative humidity of the atmosphere inside packs of these two commodities. The figure for raw meat is well above the limit for *Pseudomonas-Achromobacter* organisms; the figure for cured meat is below it, which is the reason why a different microflora develops on cured meat. The atmospheric humidity, under our conditions, despite large fluctuations seldom exceeds the E.R.H. of the meats. Consequently, these dry on exposure to the atmosphere, the raw more than the cured.

The Australian work indicated years ago that when beef was chilled, sufficient drying could occur to exert an important delay on the spoilage flora, as Fig. 1 shows. It was also found that bacterial growth accelerated when a partly dried surface was later covered. It was shown that growth stopped on exposed muscle tissue when the water content had fallen from the normal value of near 300 to about 100 g of water/100 g of dry matter (Scott & Vickery, 1939, Tables 10–13); and that the latter figure, at the relevant temperature, corresponded to the E.R.H. of 95% found to be limiting for isolated bacteria in laboratory experiments. Haines & Smith (1933) had earlier pointed out that drying is especially important on surfaces backed by connective or fatty tissue, to which water cannot readily diffuse from below. These observations indicated a great importance of partial surface desiccation in delaying spoilage.

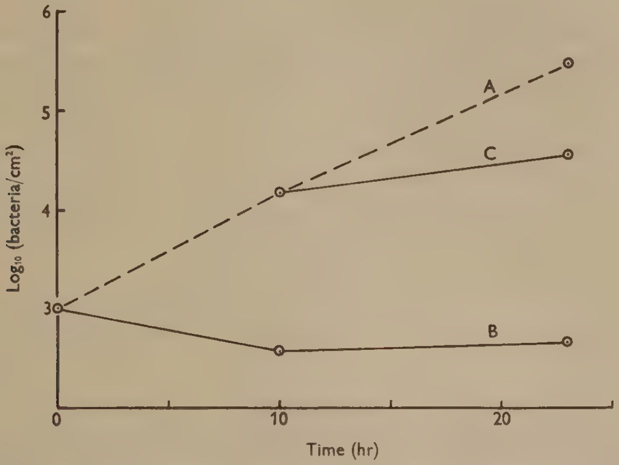

Fig. 1. The effect of surface evaporation on bacterial growth at 5° on lean meat in a commercial chiller. A, covered throughout; B, exposed throughout; C, exposed after 10 hr.
(Data of Scott & Vickery, 1939, Table 11).

There must, however, be reservations in applying this experience to prepacked meats. The observations applied strictly to the surface tissue (i.e. a layer roughly 1 mm thick), which is all that matters with an intact piece of chilled meat, the psychrophilic bacteria being virtually confined to this zone. But with meat that is cut and repacked in pieces, many of the surface bacteria become distributed between the pieces and will not be affected by drying at the surface of the mass. Again, the drying in the Australian experiments occurred mainly while the carcase was warm. With meat cooled before packing, as is usual, covering would make much less difference to the rate of evaporation. Further, in cutting meat for packing the aim is to minimize connective and fatty surfaces and expose the maximum of cut muscle surface, which is less readily affected by drying; with muscle slices in air-filled jars (Kitchell & Ingram, 1960) it was incidentally noted that quite large fluctuations in wetness of the surface did not affect the growth rate of bacteria on it, confirming the earlier observations of Haines & Smith (1933). However, a smaller degree of dryness might limit growth in an anaerobic package.

Nevertheless, the principles involved remain valid and, wherever wrapping causes a significant decrease in loss of weight, as is frequently reported, there must be significantly less surface drying. One might thus expect, as a result of the increased humidity, a shorter life with packed meats than with unpacked, more so with raw than with cured meats. Packing is obviously not recommended, except perhaps in a wrapper highly permeable to water vapour, for a product which depends on surface dryness for its stability: this is the case with dry-plucked uneviscerated poultry where, as normally exposed open for sale, evaporation from the intact skin prevents it from becoming wet enough to develop a bacterial slime; much the same applies to washed or cooked vegetables.

Correspondingly, a shorter life might be expected with a water-impermeable than with a permeable film. Tables 1 and 2 illustrate the opposite, suggesting that differences in permeability (at low levels) are less important for water vapour than for other gases. To demonstrate the effect of humidity clearly one requires experiments in which there are maintained, over the meat, atmospheres with definite constant relative humidities and otherwise identical composition, with confirmatory

Table 1. *The permeabilities of two types of wrapping film to water, oxygen and carbon dioxide*

(From Report, 1957. The figures used do not refer to samples of film actually used and should be regarded as a guide to comparative performance)

Film*	Permeability† to		
	Water at 25° (g)	Gas at 21° (mg)	
		O_2	CO_2
A	40–80	190	1200
B	10–30	0·2	1·2

* Films: A, modified polyethylene; B, vinylidene chloride-vinyl chloride co-polymer.

† Permeabilities: g or mg/24 hr/m²/mm thickness/cm of Hg, determined at relative humidities of 0% and 50% for A and B respectively.

Table 2. *The effect of the permeability of the wrapping film on the composition of the internal atmosphere and on the development of spoilage bacteria for packed chilled poultry*

(From Shrimpton & Barnes, 1960)

Holding time at 1° (days)	Film*	Gas concn (% v/v)		Viable counts† (10^5/cm²)
		O_2	CO_2	
0	A	20·8	0·2	1·5
	B	15·1	4·4	1·6
8	A	20·2	0·5	49
	B	6·5	6·2	5·2
12	A	17·1	2·1	2,800‡
	B	2·8	9·1	66
14	A	20·3	1·0	§
	B	3·9	9·3	75
16	A	§	§	§
	B	6·7	10·0	580‡

* Films: A, modified polyethylene; B, vinylidene chloride-vinyl chloride co-polymer. † Viable counts at 20°. ‡ These carcases had developed definite 'off' odours. § Tests not done.

observations (on the Australian model) of the amount of water remaining in the surface layers. Such experiments are not easy to arrange in film packs and no investigator has yet faced the difficulties involved.

4. THE COMPOSITION OF THE INTERNAL ATMOSPHERE

For practical reasons, packers are interested in the relation between the vacuum applied and the behaviour of the pack, but this gives a superficial view of the problem. The partial pressures of the gases of the internal atmosphere depend on its composition as well as its pressure, and both of these may change with time. At least it should be understood at the outset that, with the vacuum packing machines used commercially, an oxygen partial pressure of at least several mm of Hg is certain to remain, and this level will thereafter rise or fall according to the circumstances.

(a) *Oxygen*

The influence of the wrapping material on the penetration of oxygen, and hence on the microbiological picture, may conveniently be illustrated by experiments with poultry meat which, as already explained, can be packed with or without air. Table 2 shows that with a wrapper of lower oxygen permeability a longer time elapsed before production of the putrid smells characteristic for spoilage of this product when stored at chill temperatures. Similarly, Carlin, Holl & Walker (1957) observed that cut up chicken had a slightly shorter life if packed loose in permeable LSAD-30 'Cellophane' (E.I. Dupont) than if vacuum-packed in impermeable 'Saran' (Cryovac Co.).

Such experience has been interpreted as due to the lower content of oxygen inside the impermeable package: for example, this was suggested by Wells, Spencer & Stadelman (1958) who observed a longer life in evacuated polyvinyl chloride bags than in similar unevacuated bags. The spoilage of chilled poultry meat is normally caused by the development of pseudomonads, which cause putrid smells and surface sliminess when their numbers reach 10^7–10^8/cm^2 on the surface of the carcases (Ayres, 1959). Because the numbers initially are comparatively small a considerable degree of multiplication must take place before spoilage, and during this there is a selection in favour of nonpigmented strains (Thornley, Ingram & Barnes, 1960). Such organisms are 'aerobic': that is, their multiplication is much slower anaerobically than in the presence of air. When packed so that the oxygen supply is restricted their growth is hindered and they are overgrown, at least temporarily, by organisms more tolerant of such conditions, the characteristic spoilage being correspondingly delayed. These relations are illustrated in Table 3, which refers to the same experiment as Table 2. In the polyethylene wrap, in which the oxygen concentration remained throughout close to that in air, spoilage followed the course normal for unwrapped carcases, nonpigmented *Pseudomonas* spp. becoming dominant. In the vinylidene chloride-vinyl chloride co-polymer wrap, the lower oxygen content is reflected in the preponderance, instead, of organisms which (though they have still to be identified precisely) have been shown to grow well under anaerobic conditions.

Table 3. *The influence of the nature of the wrapping film on the proportions of different types of bacteria growing on wrapped chilled poultry at 1°*

(From the unpublished observations of E. M. Barnes, based on 3 carcases at each level)

Film*	Holding time (days)	Viable count at 1° (/cm²)	No. of colonies examined	Proportions (%, approx.) of types† a	b	c	d	e	f
A	0	$2{\cdot}7\times10^4$	22	20	55	5	20	0	0
	8	$4{\cdot}9\times10^6$	21	5	90	5	0	0	0
	12	$2{\cdot}8\times10^8$	28	5	80	0	5	0	10
B	0	$2{\cdot}7\times10^4$	22	5	60	20	10	0	5
	8	$5{\cdot}2\times10^5$	23	10	30	5	40	0	12
	12	$6{\cdot}6\times10^6$	26	0	5	0	70	30	0
	14	$7{\cdot}5\times10^6$	27	0	5	0	60	35	0
	16	$5{\cdot}8\times10^7$	26	0	40	0	51	0	11

* Films: A, modified polyethylene; B, vinylidene chloride-vinyl chloride co-polymer.

† Types: a, green *Pseudomonas* spp.; b, nonpigmented *Pseudomonas* spp.; c, *Achromobacter* spp.; d, atypical (brown) flavobacteria; e, Gram-positive cocci, probably faecal streptococci; f, not identified.

These considerations appear to account, in general terms, for the extension of storage life brought about by the impermeable wrapper. Similarly, practical trials with chilled beef have shown that its spoilage can be delayed if the meat is kept in an atmosphere of '100%' nitrogen. It would appear that exclusion of oxygen hinders the spoilage flora. Similar considerations might apparently apply to casual contaminating organisms (e.g. moulds and 'wild' yeasts), which are mostly aerobes.

If, however, one scrutinizes the actual concentrations of gases inside the packs, this explanation may look less satisfying. In the experiments of Table 2, even in the impermeable wrapper, an oxygen concentration of at least several percent, at near atmospheric pressure, remained throughout. It is known that a partial pressure even as low as the order 10^{-2} atm, corresponding to oxygen concentrations well below 1% at atmospheric pressure, suffices with many organisms to saturate the aerobic systems and maintain respiration and growth rates at the level in air (cf. Table 4). The partial pressure necessary is probably smaller the lower the temperature (e.g. for yeast respiration the limiting values were 4·5, 1·0 and 0·3 mm of Hg at 27°, 16°

Table 4. *The partial pressures of oxygen needed to sustain maximum rates of growth and respiration of various bacteria*

(Calculated approximately from data of Weinfurtner, Uhl & Ott, 1960)

Species	Incubation temp. (°C)	Partial pressure (atm) of O_2 for maximum: Growth	Respiration	Oxygen consumed (10^{-14}g /cell/hr)
Clostridium tyrobutyricum	37	0·003	—	—
Lactobacillus pastorianus	25	0·02	0·01	2
Escherichia coli	37	0·01		
	25		0·01	3
Acetobacter spp.	25	0·005–0·04	0·03	80

and 5°, respectively; Winzler, 1941). Though it has still to be proved that the micro-organisms on meats respond in the same way, observations like these raise the suspicion that a large, even major, part of the inhibition in an impermeable wrap might be due not to lack of oxygen but to an increased concentration of carbon dioxide such as the data of Table 2 reveal.

(b) *Carbon dioxide*

It was shown by Haines (1933) that carbon dioxide is effective against the spoilage bacteria of fresh meats. It was proved by Haines (1933) and by Coyne (1933) that the inhibition is specific, and does not depend primarily on an increased acidity caused by solution of carbon dioxide in the medium; although the pH change may sometimes be great enough to add to the inhibition it is seldom so in meats because of their high buffering power (cf. Fig. 4).

Though small concentrations of carbon dioxide may stimulate, the optimum depending on species, medium and temperature, beyond this point there is an

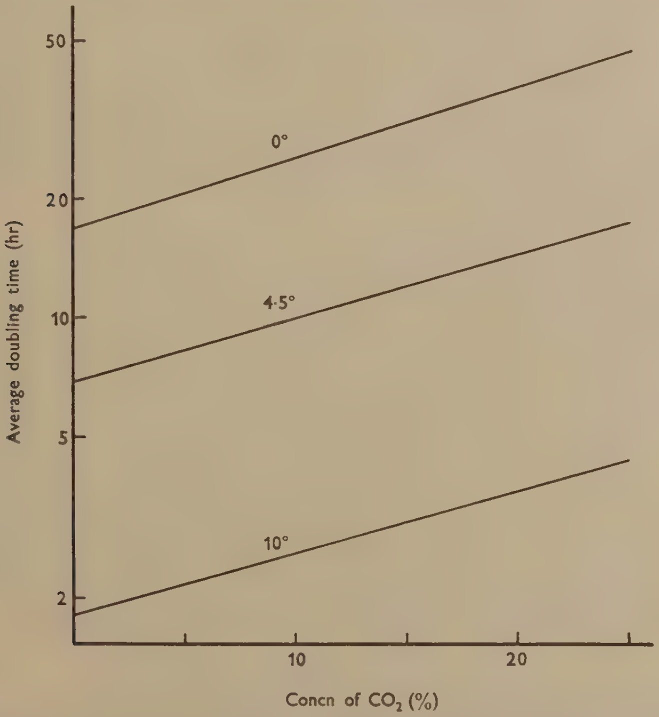

Fig. 2. The relationship between the carbon dioxide concentration (atmospheric pressure) and the average doubling time of micro-organisms growing on packed poultry meat at various temperatures. (From the data of Ogilvy & Ayres, 1951).

inhibitory effect, increasing with the concentration. Correspondingly, in a thorough investigation with poultry meat, Ogilvy & Ayres (1951) observed that the delay in spoilage increased steadily with carbon dioxide concentration up to at least 25% (Fig. 2). But, although the inhibitory effect rises with concentration, high concentrations of carbon dioxide may be undesirable because they change the colour of the meat. In the chilled beef trade, concentrations of about 20% are associated with a defect called 'gas stain' (Brooks, 1933). The limit with poultry (red meat?) is about 25% (Ogilvy & Ayres, 1951). With bacon, where the meat pigment is 'fixed' by nitrite, 100% can be tolerated (Callow, 1934).

Concentrations of carbon dioxide like those recorded in Table 2 could clearly be expected to have a significant effect. It seems conceivable that, because of the smaller volume, a greater partial pressure of carbon dioxide accumulated in the evacuated bags used by Wells *et al.* (1958) than in the unevacuated. and that this and not the lower oxygen content which they suggested, might have been the reason for the delayed putrefaction in the evacuated bags; these workers (like most others) unfortunately did not record even the internal gas concentrations. It is obviously not easy to disentangle the respective effects of oxygen and of carbon dioxide, especially since among the common packaging films the variations in permeability to these two gases go broadly in parallel (cf. Table 1; and Hannan, 1962).

In its action against a particular species, it was clearly shown by the earlier workers (Coyne, 1933; Tomkins, 1932) that carbon dioxide inhibits particularly effectively at low temperatures. More lately, Ogilvy & Ayres (1953) have confirmed that keeping under carbon dioxide extends the storage life of frankfurters much more at 30°F than at 50°F (cf. Fig. 2): such an observation, however, also involves the selective action of the gas.

The inhibitory action of carbon dioxide is distinctively selective, a fact evidently likely to influence the nature of the organisms surviving in a pack. Moulds are sensitive (Tomkins. 1932; Hays. Burroughs & Warner, 1959), yeasts comparatively resistant (Ingram, 1958). Among bacteria lactobacilli, and to a lesser extent Lactobacteriaceae generally, are among the resistant forms, and it is noteworthy that lactobacilli (unspecified) are commonly reported to occur on vacuum packed meats (fresh meats—Halleck, Ball & Stier, 1958; frankfurters—Ogilvy & Ayres, 1953; Alm *et al.* 1961; bacon—Hansen, 1960; Cavett, 1962). The importance of the selective nature of inhibition by carbon dioxide was realized at the outset by Haines (1937). Writing in relation to storage of chilled beef in carbon dioxide, he said: ". . . successful application . . . depends . . . on the happy chance that the flora capable of growth at low temperatures (Achromobacter, Pseudomonas) is susceptible to this gas, while the more resistant organisms do not as a rule grow below about 5–10°". These penetrating remarks apply equally to vacuum packed fresh meats.

5. METABOLIC EXCHANGES WITHIN THE PACK

In the preceding section, the concentrations of oxygen and of carbon dioxide existing in the pack were taken as points of reference. But these concentrations, as briefly noted earlier (see also Tomkins, 1962), themselves depend on a balance between metabolic exchanges of the contents and diffusion through the wrap, and it is now time to consider the effects of metabolism.

Virtually nothing is published on this aspect of the subject in relation to meat products, but some information has been obtained in experiments with packed bacon. In these, attention was centred on the influence of metabolic exchanges by

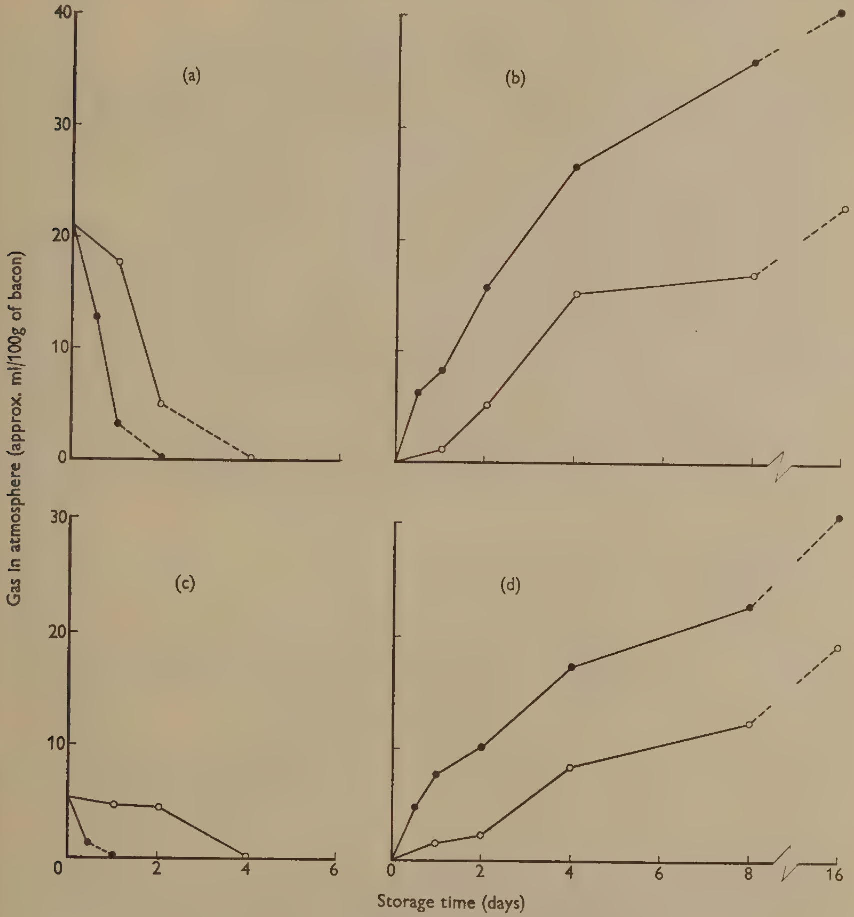

Fig. 3. Oxygen absorption (a and c) and carbon dioxide production (b and d) from Wiltshire bacon stored in cans at 15° (open circles) or 37° (full circles) and packed in air (a and b) or 5% oxygen in nitrogen (c and d).

the product, excluding complications introduced by diffusion through the wrapper: consequently the bacon was sealed in cans. Further, to make it possible to remove gas samples adequate for the simple analytical methods used, a large headspace was left in the cans. This headspace was filled with different mixtures of oxygen and nitrogen, or carbon dioxide. Details will be given elsewhere—brief comments must suffice here. Fig. 3 indicates the exchanges of oxygen and carbon dioxide in some of these cans. While the proportions should be reliable, for reasons mentioned earlier the absolute quantities are very approximate, but they serve to indicate orders of magnitude.

(a) *Oxygen consumption*

Fig. 3 shows that, even with the air pack, all the oxygen was consumed in 2–4 days at 15° or 1–2 days at 37°. The rates of consumption were not very different in 5% oxygen, confirming the earlier suggestion that reduction of oxygen concentration to such levels may have little effect and suggesting that a good vacuum will be required to produce any effect—at least with bacon. The indicated rates of consumption are of the order of 0·1 ml of O_2/g of bacon in 1 day at 37° or in 2 days at 15°.

With this information one can begin to estimate the degree of permeability which would be needed to maintain the conditions inside a vacuum pack, by comparing the total oxygen consumption with the area of wrapper. In estimating oxygen consumption it is necessary to make assumptions (cf. below) about the effective weight of bacon which is in a position to respire freely; the effective area of the wrapper, likewise, is not precisely ascertainable and the calculation can only be approximate, as shown by the following example. Take as an example 240 g (*c.* 8 oz) of bacon at 15° in a flat bag 10×25 cm, and assume that this 240 g of bacon respires at the same rate as that in the cans. An indicated consumption of 0·05×240 ml of O_2/day is then to pass through 500 cm² of wrapper, corresponding roughly to a permeability of

$$\frac{0{\cdot}05\times 1{,}000\times 240}{500\times 24} = 1 \text{ mm}^3 \text{ of } O_2/\text{cm}^2/\text{hr}$$

$$= 350 \text{ mg/m}^2/24 \text{ hr (cf. Table 1)}$$

The result is the sort of datum desired by the manufacturer of the wrapping material. A film of greater permeability, with this particular product and temperature, will give a pack into which oxygen diffuses more quickly than it can be consumed, so that a vacuum pack will gradually become more oxygenated. Given the permeability and thickness of the film, it is possible to estimate the difference in oxygen pressures inside and outside which corresponds to this rate of diffusion; this will indicate the internal concentration at which the system would reach equilibrium (cf. Tomkins, 1962).

The oxygen consumed should represent the joint respiration of the meat tissue and any bacteria on it. Fig. 3 suggests an oxygen consumption of approximately 0·2 ml/g of bacon in 4 days at 15°. For bacon slices not more than 4 mm thick and in air at 15°, Brooks (1936) observed a rate of 15×10^{-5} ml of O_2/min/ml of lean bacon tissue, which corresponds roughly to 0·9 ml/g in 4 days. Since about half the bacon in the cans was fatty tissue, which probably has a much lower respiration/unit weight than lean, the calculated value of 0·9 might be roughly halved, which would make it only two or three times greater than the observed value. This is on the assumption that all the bacon in the can was able to respire freely, which is certainly not justified as it was packed into a fairly tight roll: estimating that perhaps one third of it was freely exposed to the internal atmosphere the indicated rate of oxygen consumption roughly matches that observed. (It may be noted, in passing, that the rate of oxygen consumption is known to be different between different meats, and to be very sensitive to change in pH and temperature: cf. Urbin & Wilson, 1961). Turning now to respiration of the bacteria, Martin (1932) found that the average uptake of oxygen from air by a single cell of *Escherichia coli* at 37° was 2–9·5, or say 5×10^{-12} ml/min (*c.* fifteen times the value in Table 4). It was found that during the first two days the bacon stored in air at 37° carried roughly 10^7 bacteria of various kinds/g. This suggests an oxygen uptake by the bacteria of the order of 5×10^{-5} ml/min/g of bacon, i.e. about 0·15 ml/g in 2 days, a figure to be compared with an observed oxygen consumption at 37° (Fig. 3) of about 0·2 ml in 2 days. Thus, for the bacteria too, the calculated value is about equal to the observed, though the data refer to different kinds of bacteria. The discrepancies are not surprising in view of the very rough nature of the analysis, which can only be improved by more precise investigations. The significance of these calculations lies in the indication that the oxygen consumption of the tissue, and that of the bacteria, were both of the same order as the observed rate of oxygen consumption. This means that with bacon like that used here the influence of both the tissue itself and of its bacteria must be equally considered in interpreting atmospheric changes in the package: and, by analogy, the same might be true for other biochemical changes.

The importance of such considerations can be illustrated by contrasting examples. Take the case of cooked meat: assume (in the absence of measurements) that it has lost all power to consume oxygen, and that it is wholly free from bacteria, so that its total oxygen consumption is nil; a wrapper totally impermeable to oxygen will then be required to prevent a gradually increasing concentration of oxygen in the pack. Similarly, a bacon carrying few bacteria should require a less permeable wrap than one with many bacteria, if discolouration by oxidation is to be prevented. Unfortunately, as the foregoing should have made evident, the basic data needed to put such considerations on a reliable numerical basis have still to be obtained.

(b) *Carbon dioxide production*

Exactly similar considerations apply to carbon dioxide and, as Fig. 3 indicates, the quantities given off are of the same order as those for oxygen consumed. Plastic films are, however, generally several times more permeable to carbon dioxide than to oxygen, as Table 1 has illustrated. Thus a film of permeability sufficient to maintain aerobic conditions inside a pack should usually allow the carbon dioxide produced to escape. If, on the other hand, a film of low permeability is chosen to exclude oxygen, carbon dioxide may continue to be produced: and if the permeability were too low it would be unable to escape quickly enough, gradually destroying the vacuum and, with it, that close fit of the wrapper which is usually a desired feature in a vacuum pack.

Little is known about the capacity of meats to produce carbon dioxide, and the origin of that produced anaerobically from bacon is an interesting question. There is no obvious reason to think that it comes from the meat, where anaerobic respiration is supposed to lead simply to lactic acid. It might be produced by lactic acid bacteria, though our experience suggests that most of those on this bacon were homofermentative: Allen & Foster (1960) and Cavett (1962) also found gas producing strains on similar products. Conceivably carbon dioxide might be produced anaerobically in an oxidative process with nitrate or nitrite as the oxygen donor.

(c) *General considerations*

The observations of Fig. 3 establish the occurrence, in the cans of bacon, of an initial aerobic phase followed by an anaerobic phase of indefinite duration. Obviously conditions in these experimental cans were very different from those in vacuum packs, the absolute quantity of oxygen being much higher in proportion to the bacon than it would be in a vacuum film pack, so that the aerobic phase might have been unduly prolonged. In a film pack, on the other hand, some oxygen leaks in to prolong the aerobic phase; it may be added, in passing, that there might conceivably remain appreciable amounts of oxygen dissolved in the meat. Further, the aerobic phase must have been relatively short with the bacon used in the experiments of Fig. 3, because it began with the heavy bacterial load normal with matured Wiltshire bacon (cf. Ingram, 1960). With cooked meats, for example, a slight leakage of oxygen through the film, or an imperfect vacuum initially, might suffice to maintain the aerobic phase for a long time.

One might also expect aerobic conditions to persist longer in the outer than in the central portions of the pack, the former being nearer the source of oxygen diffusing in; the converse should apply to carbon dioxide.

The gas relations in film packs will have to be established by direct measurement, and the microbiological differences reported between different experimenters may well be explicable by unrevealed differences in this sort of respect.

6. The Nature of the Microflora

It should by now be understood that the gas exchanges depend on the magnitude and nature of the microflora, and themselves react on it. Thus, wherever there is an aerobic *Pseudomonas-Achromobacter* spoilage flora, similar considerations might apply as to chilled eviscerated poultry. This is notably the case with white fish: Coyne (1933) indeed demonstrated the inhibitory action of carbon dioxide on similar organisms from fish. With fresh meats likewise, it seems certain that the chilled storage life could be extended by vacuum packing if some means could be found to prevent the undesirable colour changes caused thereby.

The *Pseudomonas-Achromobacter* flora is characteristic of temperatures of 0°–5°. At higher temperatures different conditions may arise. With poultry the flora becomes less strictly aerobic, more scope being given to facultative anaerobes like coli-aerogenes bacteria and streptococci (E. M. Barnes, unpublished observations; there have been similar indications for other meats), so that deprivation of oxygen is less effective in delaying growth. Moreover, the effectiveness of carbon dioxide on individual species is less at higher temperatures, as noted above. For these reasons impermeable wrappers do not seem likely to delay spoilage much unless the packets are kept cool.

One might add that entirely different results would again be expected with the dry-plucked uneviscerated poultry carcases of traditional British practice. Spoilage here is caused by an anaerobic production of H_2S in the gut (Barnes & Shrimpton, 1957). Packing in an impermeable wrapper would seem likely to accelerate rather than delay this, which is a second reason against wrapping this type of bird.

Again, quite different considerations apply with, for example, bacon. The flora consists largely of micrococci and lactobacilli. The former are mostly well able to reduce nitrate contained in the bacon, if deprived of oxygen, and the latter need little oxygen and are not sensitive to carbon dioxide. It is doubtless for such reasons that the vacuum packing of bacon does not as a rule extend its storage life much, though it apparently gives some protection against moulds, which often appear after a packet has been punctured (perhaps because of the unusual acidity, cf. below).

No distinction has hitherto been made between fat and lean tissue of meat although it is probable that the flora of the two might be significantly different and respond differently to packing, in which case the proportions of fat and lean in samples would influence the result. Further, packing may select entirely accidental elements of the flora, the sporadic occurrence of which may be unsuspected because these organisms are insignificant under normal circumstances—for instance, lactobacilli cannot be selected unless meat has been contaminated with them beforehand, and if such contamination is lacking the picture will be different. Such accidents may be another reason for discrepant observations by different workers.

(a) *Succession in the microflora*

A regular series of changes in a flora, where each is determined by an alteration in conditions brought about by the preceding stage, is called by ecologists a 'succession'. The changes in the internal atmosphere caused by the metabolism of the contents of the pack will plainly react on the microflora in this kind of way.

For example, in the case of poultry loosely packed in an impermeable wrap, the metabolism of the *Pseudomonas-Achromobacter* flora will assist the tissue respiration in promoting the decrease of oxygen and increase in carbon dioxide concentrations (shown for film B in Table 1) which prepares the way for the replacement of this flora by less aerobic species.

With the canned bacon there was a similar but more complex series of changes. The organisms dominant during the aerobic phase were replaced in the early part of the anaerobic phase by a mixture of faecal streptococci, unidentified Gram-positive cocci and lactobacilli, among which lactobacilli eventually became dominant. Analogous but not exactly similar observations have been made by Hansen (1960) and Cavett (1962) with film-packed bacon. It appears that this pattern too might be determined by the gas exchanges, the decline of the micrococci following disappearance of the oxygen, and the replacement of streptococci etc. by lactobacilli, resulting from the rising concentration of carbon dioxide to which lactobacilli are comparatively resistant.

These two examples are not ideal because in the critical early stages of storage the bacteria were probably too few for their respiration to be significant in comparison with that of the tissue. This seems especially the case with the poultry meat (cf. Table 3). Clearly, however, the same general situation would develop, if more slowly, with a substrate of low respiratory activity.

(b) *Deviations of microbial metabolism*

It is often reported that with a packed meat the type of spoilage differs from that normal for the unpacked product. One reason, obvious from the above discussion, is that different conditions in the package cause different micro-organisms to be involved: but there are additional factors due to metabolic disturbance.

First, the maximum possible number of organisms (the so-called M-concentration) appears often to be lower on packed than unpacked meats. Normally, in air, viable counts rise to 10^9 or more/cm^2 of surface and 'off' odours become evident at about the level $10^{7\cdot5}$ (Haines, 1937; Ayres, 1959, 1960). With vacuum-packed meats the ultimate level seldom exceeds 10^8 and is often lower (Halleck *et al.* 1958; Allen & Foster, 1960; Hansen, 1960; Alm *et al.* 1961); in one or two cases (Hansen, 1960; author's unpublished observations) the difference has been confirmed by direct microscopic counts. It is not clear why the upper part of the growth curve should be suppressed in this way, the metabolic basis of the M-concentration phenomena being ill understood, but evidently it is often barely possible to attain the numbers normally needed to make spoilage evident.

Further, the offensively putrid smells which signalize spoilage are largely amino compounds. Broadly speaking they are produced from meat kept in air by proteolysis, culminating in oxidative breakdown of the carbon skeletons of the resulting amino acids, during which surplus amino groups are rejected with more or less of the carbon skeleton attached. When oxygen is lacking this type of breakdown is less possible and typically putrid smells are less likely to be produced.

In addition, shortage of oxygen leads many organisms to produce acid, as is well known. This, coupled possibly with a diminished production of bases as above, leads to different pH relations. An illustration was provided in the canned bacon experiments, in which the rise of pH normal for bacon stored in air was arrested about the time when the oxygen in the can was exhausted (Fig. 4). Moreover, with a low pH, amino compounds produced are 'fixed' and not detectable by smell. For such reasons it is not surprising that packed meats often spoil by going sour rather than putrid.

In cured meats absence of oxygen might be expected to stimulate reduction of nitrate, for it is known that oxygen competes successfully with nitrate for the reducing systems of bacteria (Stickland, 1931; Lindeberg, 1958; Skerman & Macrae, 1957). Though parallel considerations might well apply to reduction of the nitrite produced (Eddy, 1958), there is little information about this and it is reasonable to wonder whether anaerobic packing might lead to accumulation of unusual quantities of nitrite. In fact, this sometimes happens: in packed bacon containing enough nitrite we have observed more than 1,000 mg of $NaNO_2$/kg; and we have private reports of even twice this quantity. Nitrite is toxic, and the law applies restrictions of a few hundred mg/l. It is necessary to define the circumstances in which the objectionably high concentrations arise.

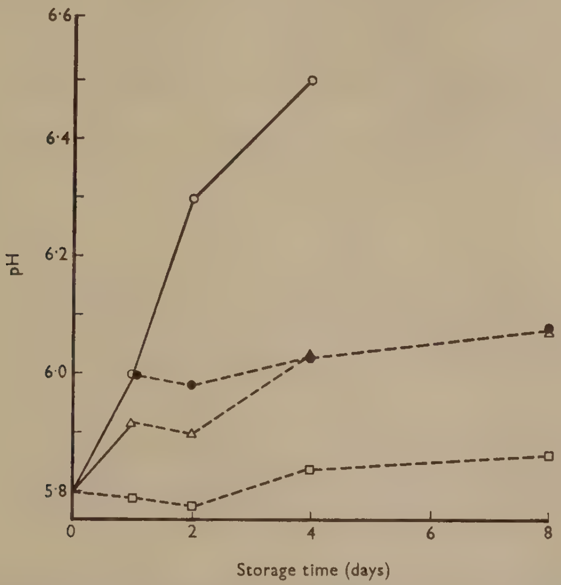

Fig. 4. The effect of composition of the atmosphere on the pH changes in Wiltshire bacon packed in cans and stored at 15°. Packs: open, open circles; closed with air, full circles; closed with 5% oxygen in nitrogen, triangles; closed with carbon dioxide alone, squares. Oxygen still present, continuous lines.

These comments, crude as they are, suffice to suggest that packing may alter the metabolism of spoilage bacteria in various important ways. Time will doubtless bring others to light. One would wish to know, for example, whether staphylococci can produce enterotoxin under the conditions inside a package.

(c) *General*

The foregoing at once provides explanations for the observation (e.g. by Hansen, 1960; Cavett, 1962) that with a prepacked meat there is not the usual correlation between spoilage and bacterial count, spoilage often becoming evident only after the number of organisms has been at its maximum for some time. This maximum number may be rather low. Under its limit, there may be successional changes in the nature of the micro-organisms, and successive groups may produce mutually neutralizing biochemical changes (as in Fig. 4). The activities even of the typical spoilage organisms may be hindered. It is scarcely surprising that the usual relation between spoilage and numbers of bacteria should disappear.

7. CONTAMINATION

As regards contamination, the effect of packaging cuts both ways. It appears beneficial in preventing the casual introduction of organisms from the air or by contact during storage of the product. On the debit side the extra handling introduces additional risks, as already mentioned. The balance of advantage depends so much on the circumstances of normal handling versus packing, and on the particular micro-organisms in question, that extensive surveys are needed before it will be possible to decide what general advantage packaging gives in this respect (cf. Eddy & Ingram, 1962).

These influences of packing on contamination of the meat may be considered in two somewhat different connections—in relation to spoilage or to hazards of food poisoning.

(a) *Effects on spoilage*

With products like fresh meats, sausages or bacon, which already carry rather large numbers of spoilage organisms, contamination at an intermediate stage is probably insignificant, though this is not proved. The contaminating organisms are relatively few, and mostly not of a kind which establish themselves as significant elements in an already developing spoilage flora. Under these circumstances, any difference in contamination caused by the package seems likely to be unimportant. Likewise with products of considerable inherent stability, like sliced salt beef, the influence of contaminants, and hence of packing upon them, seems likely to be unimportant.

With cooked meats and other highly perishable products of low microbial content the situation appears very different. Their surface offers an open field for any adventitious organisms, and the growth especially of mould contaminants from the air seems probable. Wrapping will help to prevent this, especially if oxygen is withdrawn. Here the nature and number of the organisms introduced during packing are likely to have a decisive effect on the behaviour of the pack. Thus with these products the general hygiene of the packing and earlier operations is important.

It might be supposed that with anaerobic packing spoilage by clostridia would be unusually likely, but this is less so than one might think. Conditions are in any case anaerobic in the centre of any piece of raw meat more than a few mm thick (Barnes & Ingram, 1955). With such products packing would make a significant difference only if most of the contaminating anaerobes were on the normally oxygenated surface and, unfortunately, it is not known whether such a situation commonly arises, e.g. through faecal contamination during butchering. With cooked products into which oxygen penetrates more readily, packing might perhaps make more difference. In either case cool temperatures are an effective safeguard (but see Schmidt, Nank & Lechowich, 1960).

(b) *Relation to food poisoning*

The same general arguments may be applied to food poisoning bacteria. The development of pathogens accidentally introduced seems equally likely to be hindered by the 'population pressure' of an already established flora. There is evidence of this in the observation that *Staphylococcus aureus* introduced at a level about 10^3/g into anaerobic packs of normal bacon multiplied only up to levels of 10^4–10^5/g, but up to levels exceeding 10^7/g if the relatively abundant spoilage flora was first decimated by irradiation (Fig 5; this experiment has been repeated under various conditions). Accordingly, in so far as the method of packing weakens the spoilage flora more than the pathogen, it seems possible that packing might encourage the more frequent survival of the pathogen. This was the principle behind the earlier suggestion (Ingram, 1960) that staphylococci might conceivably be more apt to develop in packed than on open bacon. Similarly, it has been shown that salmonellae multiply further on poultry if the normal flora is suppressed by tetracycline antibiotics, to which the pathogen is comparatively insensitive (Hobbs *et al.* 1960). It seems that suppression of the poultry spoilage flora by anaerobic packing might perhaps have a similar effect.

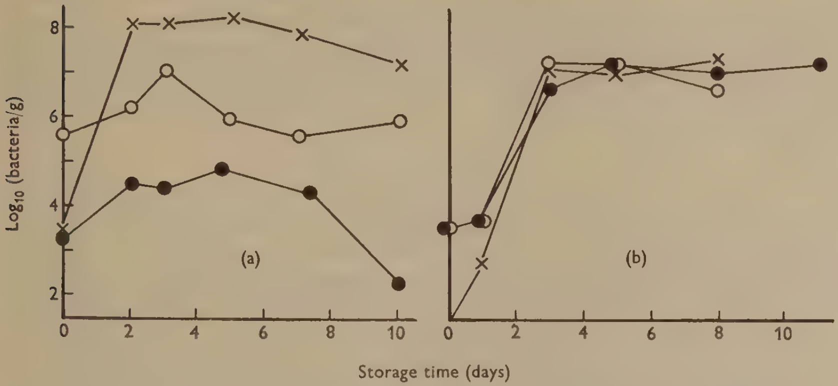

Fig. 5. The competitive inhibition of *Staphylococcus aureus* by the normal flora of packed Wiltshire bacon stored anaerobically at 25°. Crosses, lactobacilli; open circles, micrococci; full circles, staphylococci (introduced initially). Bacon not irradiated (a); bacon irradiated before inoculation (b).

Where the natural flora is small or absent, as in cooked meats, it is apparently essential to prevent access of relevant pathogens during packing. If this cannot be achieved by hygienic precautions, a pasteurizing treatment after packing may become necessary—plastic films are now available which can withstand sufficient heating. The heating cannot, however, readily be sufficient to guarantee destruction of the spores of *Bacillus* and *Clostridium* spp. if these have been admitted to the product.

Of course, the pathogens will only develop on compatible substrates: for instance, salmonellae and clostridia do not grow on well cured products, whereas staphylococci do, being salt tolerant. These habitual relations continue broadly to operate, but it is worth remembering that they may be significantly modified in a package. For example, the observation that staphylococci grow down to E.R.H. 86% aerobically but only down to 90% anaerobically (Scott, 1953) suggests that a salt:

moisture ratio ('brine concentration', cf. Hankins, Sulzbacher, Kauffman & Mayo, 1950) of only about 12%, instead of more than 15% would be needed to inhibit staphylococci if a salted meat were packed without oxygen. Though this difference is not great it suffices to bring such control of staphylococci within the range of practical possibility. Further, the lower pH and increased partial pressure of carbon dioxide might well have supplementary influences, though this is not proved. Similarly, the tolerance of staphylococci to nitrite is much lower under anaerobic than under aerobic conditions (Castellani & Niven, 1955), and it thus seems probable that at the low oxygen tension and low pH likely in a vacuum pack sufficient nitrite might often be present to prevent their growth.

8. Conclusions

To conclude, it should be emphasized that the object of this paper has been to illustrate principles. In order to do this, appropriate experimental data being for the most part lacking, it has frequently been necessary to speak in general terms or to use scanty or partial evidence. It seemed worthwhile to tolerate these shortcomings for the sake of illustrating principles now, in the hope of abating the confusion which is accumulating about the effects of packing meats. On the one hand are authors who claim, in more or less general terms, that vacuum packing makes no difference to the keeping quality of meat products (e.g. Leistner, 1956, 1957; Linderholm, 1960); on the other are those who do report a difference (e.g. Halleck *et al.* 1958; Allen & Foster, 1960; Brown & Schmucker, 1960); while even recent writers (e.g. Alm *et al.* 1961) continue to pass on the contradictions uncritically.

This paper should have shown that it is wrong to generalize over a variety of meat products. The effect of packing may depend not only on permeability of the wrapper to oxygen, carbon dioxide and perhaps water, but equally on the exact kind of meat and on the nature and number of the bacteria it happens to carry. If any generalization is found possible, it is likely to be among products with a similar spoilage flora. Further, this paper should have shown that there is little of comparative value in publications from which it is not clear how much oxygen or carbon dioxide was actually present.

For a better understanding of the microbiology, the immediate need is in fact for better methods for determining these quantities inside the packs. In addition measurements are needed of the rates of respiratory exchange by the meat product itself (data are just beginning to appear, e.g. Urbin & Wilson, 1961), and by the bacteria occurring on it. The type of analysis used for fruit packs by Tomkins (1962) is as yet quite impossible for meats because these data are lacking. It will be necessary to find the quantitative variations in respiration caused by changes in temperature, atmospheric composition and substrate composition such as occur in practice, and to find how these changes alter the other products of metabolism. To make such observations generally applicable, the identity of the relevant organisms must be established. Only if this can be done precisely enough will it become possible to trace their origins during processing; for example, one would like to know whether the lactobacilli which are so important here originate from the flora of the meat animal or are introduced adventitiously—at present this cannot be decided (cf. Ingram, 1955). There is, indeed, much to be done before microbiological observations on packed meats can be anything more than *ad hoc* empiricism.

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10. REFERENCES

ALLEN, J. R. & FOSTER, E. M. (1960). Spoilage of vacuum-packed sliced processed meats during refrigerated storage. *Food Res.* **25**, 19.

ALM, F., ERICHSEN, I. & MOLIN, N. (1961). The effect of vacuum packaging on some sliced processed meat products as judged by organoleptic and bacteriological analysis. *Food Tech., Champaign*, **15**, 199.

AYRES, J. C. (1959). Effect of sanitation, packaging and antibiotics on the microbial spoilage of commercially processed poultry. *Iowa St. Coll. J. Sci.* **34**, 27.

AYRES, J. C. (1960). Temperature relationships and some other characteristics of the microbial flora developing on refrigerated beef. *Food Res.* **25**, 1.

BARNES, E. M. & INGRAM, M. (1955). Changes in the oxidation-reduction potential of the sternocephalicus muscle of the horse after death, in relation to the development of bacteria. *J. Sci. Fd Agric.* **6**, 448.

BARNES, E. M. & SHRIMPTON, D. H. (1957). Causes of greening of uneviscerated poultry carcases during storage. *J. appl. Bact.* **20**, 273.

BROOKS, J. (1933). The effect of carbon dioxide on the colour changes or bloom of lean meat. *J. Soc. chem. Ind., Lond.*, **52**, 17T.

BROOKS, J. (1936). The oxygen uptake of pork and bacon. A factor in the preservation of the colour of bacon. *J. Soc. chem. Ind., Lond.*, **55**, 12T.

BROWN, W. L. & SCHMUCKER, M. L. (1960). The influence of low level gamma irradiation, antibiotic treatment, storage temperature and vacuum packaging on flavour and bacterial changes in cured bacon. *Food Tech., Champaign*, **14**, 92.

CALLOW, E. H. (1934). Gas storage (of bacon). *Rep. Fd Invest. Bd, Lond.*, p. 71.

CARLIN, A. F., HOLL, B. E. & WALKER, H. W. (1957). Correlation between flavour and number of microorganisms associated with eviscerated chicken treated with chlortetracycline. *Food Tech., Champaign*, **11**, 573.

CASTELLANI, A. G. & NIVEN, C. F. (Jr.) (1955). Factors affecting the bacteriostatic action of sodium nitrite. *Appl. Microbiol.* **3**, 154.

CAVETT, J. J. (1962). The microbiology of vacuum packed sliced bacon. *J. appl. Bact.* **25**, 282.

CHRISTIAN, J. H. B. (1955). The influence of nutrition on the water relations of *Salmonella oranienberg*. *Aust. J. biol. Sci.* **8**, 75.

COYNE, F. P. (1933). The effect of carbon dioxide on bacterial growth. *Proc. roy. Soc. Lond.*, Ser. B, **113**, 196.

EDDY, B. P. (1958). The effects of salt concentration and temperature on nitrite production and metabolism. In The Microbiology of Fish and Meat Curing Brines. *Int. Symp. Fd Microbiol., Cambridge* p. 87. London: H.M.S.O.

EDDY, B. P. (1961). Refrigeration in relation to food hygiene. *Proc. Hlth Congr., Blackpool*, p. 152. London: Roy. Soc. Hlth.

EDDY, B. P. & INGRAM, M. (1962). The occurrence and growth of staphylococci on packed bacon, with special reference to *Staphylococcus aureus*. *J. appl. Bact.* **25**, 237.

GREENBERG, R. A., SILLIKER, J. H., NANK, W. K. & SCHMIDT, C. F. (1958). Toxin production and organoleptic breakdown in vacuum packaged fresh meats inoculated with *Clostridium botulinum*. *Food Res.* **23**, 656.

GREENBERG, R. A., SILLIKER, J. H. & FATTA, L. D. (1959). The influence of sodium chloride on toxin production and organoleptic breakdown in perishable cured meat inoculated with *Clostridium botulinum*. *Food Tech., Champaign*, **13**, 509.

Haines, R. B. (1933). The influence of carbon dioxide on the rate of multiplication of certain bacteria, as judged by viable counts. *J. Soc. chem. Ind., Lond.*, **52**, 13T.

Haines, R. B. (1937). Microbiology in the preservation of animal tissues. *Spec. Rep. Fd Invest. Bd, Lond.*, no. 45. London: H.M.S.O.

Haines, R. B. & Smith, E. C. (1933). The storage of meat in small refrigerators. *Spec. Rep. Fd Invest. Bd, Lond.*, no. 43. London: H.M.S.O.

Halleck, F. E., Ball, C. O. & Stier, E. F. (1958). Factors affecting quality of prepackaged meat. IV. Microbiological studies. *Food Tech., Champaign*, **12**, 197.

Hankins, O. G., Sulzbacher, W. L., Kauffman, W. R. & Mayo, M. E. (1950). Factors affecting the keeping quality of bacon. *Food Tech., Champaign*, **4**, 33.

Hannan, R. S. (1962). Some properties of packaging materials which relate to the microbial flora of the contents. *J. appl. Bact.* **25**, 248.

Hansen, N.-H. (1960). Quality deterioration and bacterial growth in prepacked bacon. *Danish Meat Res. Inst. Publ.* no. 28.

Hays, G. L., Burroughs, J. D. & Warner, R. C. (1959). Microbiological aspects of pressure packaged foods. II. The effects of various gases. *Food Tech., Champaign*, **13**, 567.

Hobbs, B. C. (1953). *Food Poisoning and Food Hygiene.* London: Arnold.

Hobbs, B. C., Reeves, J. C., Garside, J. S., Gordon, R. F., Barnes, E. M. & Shrimpton, D. H. (1960). Antibiotic treatment of poultry in relation to *Salmonella typhi-murium*. *Mon. Bull. Minist. Hlth Lab. Serv.* **19**, 178.

Ingram, M. (1949). Science in the imported meat industry. II. Hygiene and storage. *J.R. sanit. Inst.* **69**, 35.

Ingram, M. (1955). Factors controlling the appearance of various bacteria in semi-preserved meats. *Ann. Inst. Pasteur, Lille*, **7**, 136.

Ingram, M. (1958). Yeasts in food spoilage. In *The Chemistry and Biology of Yeasts*, p. 603. New York: Academic Press Inc.

Ingram, M. (1960). Bacterial multiplication in packed Wiltshire bacon. *J. appl. Bact.* **23**, 206.

Kitchell, A. G. & Ingram, M. (1960). The survival of bacteria on frozen meat and their survival after thawing. *Int. Congr. Refrig., Copenhagen*, 3, 65. London: Pergamon Press.

Leistner, L. (1956). Die neuzeitliche Verpackung in der Fleischwirtschaft. I. *Fleischwirtschaft* **8**, 422.

Leistner, L. (1957). Die neuzeitliche Verpackung in der Fleischwirtschaft. II. *Fleischwirtschaft* **9**, 262.

Lindeberg, G. (1958). Levan-producing halophilic bacteria in herring brines. In The Microbiology of Fish and Meat Curing Brines., *Int. Symp. Fd Microbiol., Cambridge*, p. 157. London: H.M.S.O.

Linderholm, K. G. (1960). Bakteriologiska och hygieniska undersökningar av vacuumförpackade charkutivaror. *Nord. hyg. Tidskr.* **51**, 17.

Martin, D. S. (1932). The oxygen consumption of *Escherichia coli* during the lag and logarithmic phases of growth. *J. gen. Physiol.* **15**, 691.

Mossel, D. A. A. & Ingram, M. (1955). The physiology of the microbial spoilage of foods. *J. appl. Bact.* **18**, 233.

Ogilvy, W. S. & Ayres, J. C. (1951). Post-mortem changes in stored meats. II. The effect of atmospheres containing carbon dioxide in prolonging the storage life of cut-up chicken. *Food Tech., Champaign*, **5**, 97.

Ogilvy, W. S. & Ayres, J. C. (1953). Post-mortem changes in stored meats. V. Effect of carbon dioxide on microbial growth on stored frankfurters and characteristics of some microorganisms isolated from them. *Food Res.* **18**, 121.

Report (1957). Films Chart. *Mod. Plast. Encyl.* p.559.

Schmidt, C. F., Nank, W. R. & Lechowich, R. V. (1960). Radiation sterilisation of food. Part II. Some aspects of growth sporulation and radiation resistance of spores of *Clostridium botulinum* type E. *Food Tech., Champaign*, **14**, 43.

Scott, W. J. (1936). The growth of microorganisms on ox muscle. I. The influence of water content of substrate on rate of growth at −1°C. *J. Coun. sci. industr. Res. Aust.* **9**, 177.

Scott, W. J. (1953). Water relations of *Staphylococcus aureus* at 30°C. *Aust. J. biol. Sci.* **6**, 549.

Scott, W. J. (1957). The water relations of food spoilage microorganisms. *Advanc. Food Res.* **7**, 83.

Scott, W. J. & Vickery, J. R. (1939). Investigations on chilled beef. II. Cooling and storage in the meatworks. *Bull. Coun. sci. industr. Res. Aust.* no. 129.

Shrimpton, D. H. & Barnes, E. M. (1960). A comparison of oxygen permeable and impermeable wrapping materials for the storage of chilled eviscerated poultry. *Chem. & Ind.* p. 1492.

SKERMAN, V. B. D. & MACRAE, I. C. (1957). The influence of oxygen availability on the degree of nitrate reduction by *Pseudomonas dentrificans*. *Canad. J. Microbiol.* **3**, 505.

STICKLAND, L. H. (1931). The reduction of nitrates by *Bact. coli*. *Biochem. J.* **25**, 1543.

THORNLEY, M. J., INGRAM, M. & BARNES, E. M. (1960). The effects of antibiotics and irradiation on the *Pseudomonas-Achromobacter* flora of chilled poultry. *J. appl. Bact.* **23**, 487.

TOMKINS, R. G. (1932). The inhibition of the growth of meat attacking fungi by carbon dioxide. *J. Soc. chem. Ind., Lond.*, **31**, 261T.

TOMKINS, R. G. (1962). The conditions produced in film packages by fresh fruit and vegetables and the effect of these conditions on storage life. *J. appl. Bact.* **25**, 290.

URBIN, M. C. & WILSON, G. D. (1961). The post-mortem oxygen requirements of bovine tissue. *J. Fd Sci.* **26**, 314.

WEINFURTNER, F., UHL, A. & OTT, H. (1960). Die Bedeutung des gelösten Sauerstoffs für die Vermehrung der Mikroorganismen. *Arch. Mikrobiol.* **36**, 1.

WELLS, F. E., SPENCER, J. V. & STADELMAN, W. J. (1958). Effect of packaging materials and techniques on shelf-life of fresh poultry meat. *Food Tech., Champaign*, **12**, 425.

WINZLER, R. J. (1941). The respiration of bakers' yeast at low temperatures. *J. cell. comp. Physiol.* **17**, 263.

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THE MICROBIOLOGY OF VACUUM PACKED SLICED BACON

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1. Introduction

Wiltshire bacon is prepared by immersing sides of pork in a matured brine solution containing sodium chloride, sodium nitrate and a characteristic bacterial flora (up to 10^9/ml). The bacteria reduce the nitrate to nitrite and this combines with the myoglobin of the lean meat to give the characteristic red colour. The microbiology of brines, including bacon brine, was the subject of a symposium held at Cambridge in 1957 (Report, 1958). After brining, the sides of bacon are matured and they may be smoked. Then rashers are cut from the side of bacon as required for use. Freshly prepared Wiltshire bacon carries a heavy bacterial load, 10^5–10^6/g according to Ingram (1960).

The effect of vacuum packing on shelf life has recently been studied by several investigators. Because of the high initial numbers of bacteria, vacuum packed sliced bacon made by the Wiltshire cure has a variable but rather short shelf life. Ingram (1960) suggested that vacuum packing might give a false impression of safety because bacterial multiplication is still rapid under these conditions. Hansen (1960) studied vacuum packed bacon stored at −40°, +5° and +20°, and reported that the product stored at low temperatures had a good shelf life; but when stored at +20° it spoiled rapidly and the initial flora of micrococci was replaced by high numbers of lactic acid bacteria.

Although Wiltshire bacon is made from whole sides of pork, it is possible in the manufacturing trade to use only part of the side for bacon manufacture and to use the rest in other ways. The work reported in this paper was done on blocks 18–22 in. long taken from the side. These were very suitable for slicing and vacuum packing and were cured in fresh brine, as distinct from the old Wiltshire cure. The brines contained added nitrite as well as nitrate, and the resulting bacon generally contained only 10^3–10^4 bacteria/g.

Spoilage resulting from bacterial multiplication is assessed subjectively. The aim of this work was to describe spoilage in measurable bacteriological and chemical quantities. This problem is one of resolving a number of interdependent bacterial and chemical changes which are proceeding simultaneously. The type of spoilage occurring depends upon which of the simultaneous processes predominates.

Vacuum packed sliced bacon is a closed ecological system. There is no moisture loss through the plastic film and therefore no change from the initial sodium chloride concentration of the pack. In addition, there is no further bacterial contamination after the bacon is packed and therefore increases in bacterial numbers must be due to multiplication.

Apart from variations in the initial flora, two main factors affect the bacteriological and chemical changes within the pack during storage. The storage temperature exerts a selective effect on bacterial development and variation in it can entirely alter the type of spoilage. The other factor is the initial salt content of the bacon, which can vary owing to differences in salt uptake during brining. We have therefore investigated the effects of these two factors and have isolated and identified the main bacterial groups and studied the chemical changes which occur in the product.

2. MATERIALS AND METHODS

Storage experiments. The bacon was cured, sliced and vacuum packed at the factory. Packets used for a storage experiment were all derived from the same block. Four of these storage experiments were done; bacon with the normal salt content of 5 7% (w/v) in the aqueous phase was used in three of them, but for the fourth a block with an unusually high salt content (8–12%) was used.

Sampling. Whole rashers or parts thereof were aseptically weighed and homogenized either in 6% (w/v) salt solution or in water. This concentration of salt was preferred because it prevented sudden osmotic changes; but some of the selective culture media became unsatisfactory due to the amount of salt carried over, and this also complicated the chemical analyses. In two of the four experiments, therefore, the bacon samples were homogenized in four times their weight of sterile distilled water and further dilutions were prepared in 0·1% (w/v) peptone water.

Assessment of spoilage. After bacteriological samples had been removed the odour of the remaining bacon in the stored packet was compared with the odour of a fresh packet. When a difference was noted by three experienced workers spoilage was recorded as having started.

Culture media. Tryptone-yeast-glucose-salt agar (TYGS) (tryptone, 0·5 g; yeast extract, 0·3 g; glucose, 0·1 g; NaCl, 6·0 g; agar, 1·5 g; distilled water, 100 ml; pH 7·2) incubated at 30° for 3 days was used to enumerate halotolerant organisms, mainly group D streptococci and catalase positive cocci; the phenolphthalein diphosphate agar (PP) of Barber & Kuper (1951) incubated at 30° for 3 days permitted development of all phosphatase positive staphylococci; the egg yolk-tellurite-glycine-pyruvate agar (ETGPA) of Baird-Parker (1962) incubated at 37° for 24 hr was used to select and differentiate coagulase positive staphylococci; the thallous acetate-tetrazolium-glucose agar (TlTG) of Barnes (1956) incubated at 37° for 24 hr was employed for group D streptococci, and the acetate agar (AA) of Rogosa, Mitchell & Wiseman (1951) incubated at 30° for 3 days gave the numbers of aciduric lactic acid bacteria.

All the media, except AA, were inoculated by spreading 0·1 ml of decimal dilutions on the surface of dried plates. Medium AA was poured into plates containing 1·0 ml of the dilutions. All plates were incubated aerobically.

The colonies on TYGS, TlTG and AA were tested for catalase by spotting with 3% (v/v) H_2O_2. Other colonies were picked from replicate plates and the various bacteria (about 1,500 isolates) will be more fully described and classified in a later publication.

Chemical analyses. These were done on the rashers and homogenates remaining after the bacteriological samples had been taken. The contents of amino acids, sugars, fatty acids, sodium chloride, nitrate, nitrite and moisture were determined at intervals during storage. Details will be given in a later publication.

3. Results

The main bacterial groups isolated were: catalase positive cocci, comprising micrococci and coagulase negative staphylococci; and lactic acid bacteria, comprising lactobacilli, group D streptococci, leuconostocs, pediococci, and several types of pleomorphic bacteria and motile rods which have not yet been identified.

None of the media was fully selective and several of the main bacterial groups were recovered from more than one medium (Table 1). It should be noted that the

Table 1. *The main bacterial groups isolated during storage trials and their incidence on various media*

Group	Incidence* of group on			
	TYGS‡	PP‡	TlTG‡	AA‡
Staphylococci	+	+	(+)	−
Micrococci	+	−	−	−
Group D streptococci	+	−	+	(+)
Lactobacilli	−	−	−	+
Leuconostocs	−	−	(+)	+
Pediococci	−	−	(+)	+
Unidentified lactic acid bacteria	+	−	−	+
Yeasts	+	−	−	+

* Incidence: +, predominant group; (+), in small numbers; −, absent. ‡ TYGS, tryptone-yeast-glucose-salt agar; PP, phenolphthalein diphosphate agar; TlTG, thallous acetate-tetrazolium-glucose agar; AA, acetate agar.

catalase positive cocci were classified as in the 7th Edition of Bergey's *Manual* (Breed, Murray & Smith, 1957) into micrococci (oxidative and relatively inactive on fat and protein substrates) and staphylococci (fermentative and actively lipolytic and proteolytic). Almost all of them were coagulase negative. Coagulase positive staphylococci were not regularly isolated, but when present they were best enumerated on the selective and diagnostic medium of Baird-Parker (1962).

Chemical analyses showed that the bacon contained a complete range of nutrients to support the types of bacteria found. Besides protein and fat (and their component amino acids, fatty acids, etc.) reducing sugars were also found, i.e. about 0·5% (w/w) of glucose and 0·05% (w/w) of ribose. Nitrate and nitrite were present from the brine.

The bacteriological and chemical changes which took place during storage at 20° and 30° are summarized in Table 2.

Table 2. *The spoilage changes in bacon of normal and high salt content stored at 20° and 30° for 22 days*

Storage temperature	Salt in aqueous phase (%)	Dominant bacterial groups	Spoilage odour and time (days) when noticed	Chemical changes
20°	5–7	Micrococci Lactic acid bacteria*	Scented-sour, 15	Amino acids and fatty acids released slowly
	8–12	Micrococci Leuconostocs	Cheesy, 15	
30°	5–7	Coagulase negative staphylococci Lactic acid bacteria*	Putrid, 9	Amino acids and fatty acids released rapidly
	8–12	Coagulase negative staphylococci Leuconostocs	Cheesy, 15	

* Group D streptococci, lactobacilli, pediococci and leuconostocs.

(a) *Changes during storage at 20°*

Bacon with the normal salt content of 5–7% (w/v) in the aqueous phase showed the first organoleptic signs of spoilage after about 15 days, when scented-sour smells were detected.

The changes in the numbers of colonies counted on TYGS and TITG are shown in Fig. 1. These data were obtained from one of the blocks of normal bacon, but very similar results were obtained with the other two blocks. At first the catalase positive cocci outgrew all the other bacteria, but as the bacon aged group D streptococci became very numerous, forming about 50% of the flora after 9 days.

The micrococci outnumbered the biochemically more active staphylococci. The latter were almost entirely coagulase negative types, although the coagulase positive *Staphylococcus aureus* was detected (on ETGPA) in occasional packets. The numbers of this species were always low ($<10^3$/g) and there was no evidence of multiplication.

The nitrite content of the bacon remained at about 50 p/m during the first 6 days of storage, after which it fell steadily to a final level of 10 p/m. There was a slow release of amino acids and fatty acids up to 22 days of storage, the longest period tested.

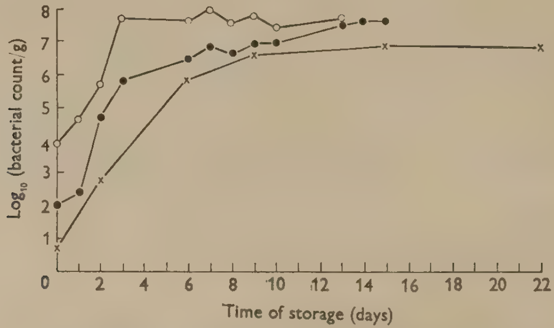

Fig. 1. The changes in the bacterial counts on TYGS and TlTG from bacon of normal salt content (5–7%) stored at 20°. Open circles, micrococci on TYGS; closed circles, *Streptococcus faecalis* on TlTG; crosses, *Strep. faecium* type on TlTG.

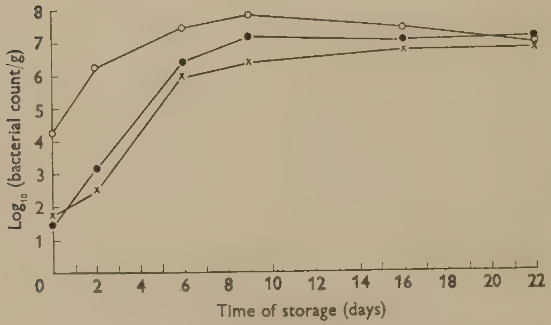

Fig. 2. The changes in the bacterial counts on acetate agar from bacon of normal salt content (5–7%) stored at 20°. Open circles and closed circles, first and second trials, respectively, both with samples homogenized in 6% salt solution; crosses, third trial with samples homogenized in water.

Fig. 2 shows the changes in the lactic acid bacteria recovered on AA during three storage trials. These bacteria were sometimes as numerous as those recovered on TYGS, but wide variations occurred in different trials. The difference between salt and water maceration can also be seen from Fig. 2: the counts were lower when water was used. This could have been due either to variations in the flora or to the effect of the water. Experience with other samples showed that aqueous homogenization generally gave lower counts on acetate agar.

The bacon with 8–12% of salt showed a considerable difference in its lactic acid flora. Streptococci and pediococci did not develop, and the catalase negative flora consisted largely of leuconostocs. Micrococci were again the dominant catalase positive organisms, and coagulase positive staphylococci were not detected. Yeasts were frequently recovered from high salt bacon and formed up to 50% of the colonies on AA.

The nitrite levels increased from 45 p/m to 60 p/m during the first 15 days of storage, due to the virtual absence of nitrite reducing bacteria. The final nitrite content at 22 days was 30 p/m. The other observed chemical changes were similar to those recorded for the bacon of normal salt content. The onset of spoilage occurred at about the same time and was marked by the development of a cheesy odour instead of the usual scented-sour smell.

(b) *Changes during storage at* 30°

The 5–7% salt bacon first spoiled at about 9 days with a putrid smell. The relative numbers of lactic acid bacteria and catalase positive bacteria were similar to those at 20°. The main lactic acid bacteria were of similar types except that unidentified motile catalase negative rods appeared at 15–22 days and reached counts of 10^5/g. There was however a big change in the development of catalase positive types. The coagulase negative staphylococci, which are much more active biochemically than the micrococci, became the major group of cocci. Products of proteolysis and lipolysis appeared much more rapidly than at 20° and the nitrite content fell steadily to 5 p/m at 22 days. The staphylococci were once again almost all coagulase negative, although they were phosphatase positive. Coagulase positive staphylococci still appeared only occasionally, but one packet had a count of 10^5/g after 6 days; this was the highest level of recovery obtained.

The high salt content had a good effect on the keeping quality of bacon stored at this high temperature. Whereas normal salt bacon was described as putrid at 9 days, high salt bacon was acceptable at this time; the next sampling date was at 15 days, when the packs with high salt content had just commenced to spoil with the development of a cheesy smell. The organisms predominating were coagulase negative staphylococci and leuconostocs. The motile rods reached counts of 10^6 g at 15–22 days. The chemical changes were similar to those in bacon of normal salt content. *Staph. aureus* was not recovered.

4. DISCUSSION

The organisms found to be most numerous in the vacuum packed sliced bacon were the lactic acid bacteria. This corresponds with the experience of other workers on vacuum packed products, both bacon (Hansen, 1960) and other meats (Allen & Foster, 1960). Group D streptococci are characteristic of the normal vacuum packed sliced bacon; leuconostocs and pediococci are probably widespread. Rod forms are also numerous, but their morphology may be greatly affected by the amount of salt and other factors in the medium. Their classification is still being studied. No obligate anaerobes have been detected in vacuum packed bacon.

The bacon also contained large numbers of catalase positive, coagulase negative cocci, including both micrococci and staphylococci as defined in Bergey's *Manual*

(Breed, Murray & Smith, 1957). In bacon stored at 20° the micrococci were more numerous than the staphylococci, whereas at 30° the staphylococci became the predominant catalase positive coccal form. These changes in bacterial flora were correlated with the organoleptic assessment of spoilage. At 20° the off flavour of normal bacon (5–7% salt) was described as scented-sour, whereas at 30° it was putrid.

Storage temperature also affected the chemical changes observed in the bacon. Lipolysis and proteolysis were slow at 20° but rapid at 30°. Proteolysis is often associated with the production of strong putrid odours, so that at elevated storage temperature such odours would mask other spoilage flavours which may develop. Proteolysis is probably caused by the catalase positive cocci; the lactic acid bacteria are unlikely to be important in spoilage at 30°.

At 20° proteolysis and lipolysis are slow and other compounds could be detected organoleptically. The heterofermentative lactic acid bacteria and pediococci which were encountered on bacon are known to produce volatile substances during fermentation (Rogosa & Sharpe, 1959; Garvie, 1960; and Pederson, cited by Breed, Murray & Smith, 1957). These substances, together with the slowly accumulating products of lipolysis, probably give rise to the characteristic scented-sour off odour in bacon allowed to spoil at normal room temperature.

Ingram (1960) also observed that the flora changed at different temperatures of storage. In his experiments, Wiltshire cured bacon was packed in cans in various mixtures of N_2 and O_2 and stored at 37° and 15°. He concluded that at lower temperatures the better adapted organisms present in large numbers in this bacon could crowd out coagulase positive staphylococci. He further suggested that in bacon specially produced with a low initial count this protective factor might be absent. Under these circumstances coagulase positive contaminants which may enter during packing might develop to a dangerous level even at moderate temperatures.

Our experience with bacon of low initial count shows that at lower temperatures (e.g. 20°) it is still the micrococci which become predominant, even though their numbers do not at first exceed 10^3–10^4 g. In agreement with Ingram (1960), we found that at 30° staphylococci develop in this bacon; but these are food spoilage rather than food poisoning types, so the protective factor still seems to operate. The coagulase positive types were of infrequent occurrence and even at 30° only one packet was found in which the number of *Staphylococcus aureus* was about 10^5/g after storage for 6 days. The organism could not be recovered from packets stored for a longer time.

Bacterial multiplication is most rapid during the first few days of storage and numbers rise to levels similar to those found in the traditional product. Numbers of all the main groups then remain steady until off odours develop. Hansen (1960) noted a similar time lag between the achievement of maximum bacterial counts and the onset of organoleptic spoilage even though his bacon was prepared differently and the brine contained sucrose. In our experiments, when the packets had spoiled the predominant groups of bacteria began to decline in numbers. Other bacteria—e.g. the motile, Gram-positive, catalase negative rods which were isolated from packets stored at 30°—continued to multiply. Thus the bacteria isolated from packets after prolonged storage may be very different from those associated with spoilage.

5. SUMMARY

Bacon with the normal NaCl content of 5–7% (w/v) in the aqueous phase spoiled at about 15 days with a scented-sour odour when stored at 20°, probably due to the combined biochemical activities of micrococci and lactic acid bacteria. The same bacon stored at 30° became putrid at 9 days, and coagulase negative staphylococci were the dominant spoilage organisms. Coagulase positive staphylococci were found only occasionally and they did not multiply at 20°, but some multiplication occurred at 30°. Other bacteria, however, reached much higher levels at 30°.

Bacon of higher NaCl content (8–12%) contained fewer lactic acid bacteria and no coagulase positive staphylococci. Its keeping quality at 20° was similar to that of the normal bacon but was longer at 30°.

6. ACKNOWLEDGMENTS

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7. REFERENCES

ALLEN, J. R. & FOSTER, E. M. (1960). Spoilage of vacuum-packed sliced processed meats during refrigerated storage. *Food Res.* **25**, 19.

BAIRD-PARKER, A. C. (1961). An improved diagnostic and selective medium for isolating coagulase positive staphylococci. *J. appl. Bact.* **25**, 12.

BARBER, M. & KUPER, S. W. A. (1951). Identification of *Staphylococcus aureus* by the phosphatase reaction. *J. Path. Bact.* **63**, 65.

BARNES, E. M. (1956). Methods for the isolation of faecal streptococci (Lancefield group D) from bacon factories. *J. appl. Bact.* **19**, 193.

BREED, R. S., MURRAY, E. G. D. & SMITH, N. R. (1957). Bergey's *Manual of Determinative Bacteriology*, 7th ed. London: Baillière, Tindall & Cox.

GARVIE, E. I. (1960). The genus *Leuconostoc* and its nomenclature. *J. Dairy Res.* **27**, 283.

HANSEN, N.-H. (1960). Quality deterioration and bacterial growth in prepacked bacon. Publication no. 28: Danish Meat Research Institute.

INGRAM, M. (1960). Bacterial multiplication in packed Wiltshire bacon. *J. appl. Bact.* **23**, 206.

REPORT (1958). The Microbiology of Fish and Meat Curing Brines. *Int. Symp. Food Microbiol., Cambridge.*

ROGOSA, M. & SHARPE, M. E. (1959). An approach to the classification of the lactobacilli. *J. appl. Bact.* **22**, 329.

ROGOSA, M., MITCHELL, J. A. & WISEMAN, R. F. (1951). A selective medium for the isolation of oral and faecal lactobacilli. *J. Bact.* **62**, 132.

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Tomkins, R. G. (1962). *J. appl. Bact.* **25** (2), 290–307.

(Symposium on The Effect of the Newer Forms of Packaging on the Microbiology and Storage Life in Various Foods: Paper IV)

THE CONDITIONS PRODUCED IN FILM PACKAGES BY FRESH FRUITS AND VEGETABLES AND THE EFFECT OF THESE CONDITIONS ON STORAGE LIFE

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1. Introduction

Prepackaging has been primarily adopted as a means to simpler and more efficient marketing and for this purpose containers made of transparent films have the great advantage of allowing the contents to be seen. Plastic films are, however, relatively impermeable to water vapour, carbon dioxide, oxygen and ethylene and hence fruits and vegetables packed in plastic bags may be subjected to conditions of higher humidity, higher concentrations of carbon dioxide and of ethylene and lower concentrations of oxygen than those to which unpacked produce is subjected.

It is usually advantageous to reduce evaporation, though films of moisture condensing on the inner surfaces of film packages are considered undesirable. The storage of certain fruits and vegetables in an atmosphere richer in carbon dioxide and poorer in oxygen than air is often an advantage, though excessive concentrations of carbon dioxide, or anaerobic conditions, can cause damage. The possibilities of using plastic containers to provide favourable conditions for storage have been widely considered and success has been achieved in certain instances. For example, the possibility of prolonging the storage life of apples and pears by the use of polythene liners and storage at low temperatures has been clearly demonstrated by Gerhardt (1955) and by Hardenburg (1956) in the U.S.A. and Marcellin (1959, 1961) in France.

Much of the available information on the properties of films and on their use in the marketing and storage of fruits and vegetables has been summarized by Nicolaisen-Scupin (1960), but there have been few attempts to consider from general principles how the conditions are established in packages and how they can be calculated. There has also been a general failure to recognize the difficulty of completely sealing packages or the effect of small leaks on the composition of the air in packages. Tomkins (1960, 1961) described a method of studying the way in which equilibrium conditions were established in packages and also attempted to assess the effect of these conditions on storage life. This paper gives further data on the way in which the concentrations of carbon dioxide, oxygen and ethylene are changed within a package, and on the way the conditions established in packs affect the storage life of prepacked fruits and vegetables.

2. MATERIALS AND METHODS

(a) *Sealing materials*

The plastic materials used in these experiments had been obtained either direct from the manufacturer or from supplying agents. No special significance need be ascribed to the source or type of the material used. Different batches of polythene of the same thickness may differ somewhat in permeability and the same probably holds for other plastic materials. No attempt is made to provide accurate figures for the absolute or relative permeability of different materials; the aim is to illustrate in a general way the difference in the conditions which can be produced in sealed packages because of differences in permeability to CO_2 and O_2. The wax used to seal the films for the aluminium trays was made by melting together vaseline, beeswax and paraffin wax to give a mixture which sets at about 40° but which was still somewhat soft at 15°. The exact composition of the wax is of no importance; any wax which has a suitable consistency and adheres to the film and the metal tray is suitable.

The adhesive polythene tape used to bind the edges of the film was originally obtained from Vicson's Ltd., of Harrow. It can now be obtained from Sellotape Products Ltd., 8–10 Paul Street, London, E.C.2.

(b) *General methods*

The conditions within sealed plastic containers were studied in the following way.

Aluminium trays (with dimensions of $41 \times 38 \times 7{\cdot}5$ cm and of 11·5 litres capacity) fitted with air sampling taps were covered with a metal sheet or a plastic film held in position by wax and bound with adhesive polythene tape. Before sealing, 5, 10, 15 or some other suitable number of fruits were placed in each tray and, except when the rate of respiration was to be measured, the free space was filled with sealed bottles. The purpose of reducing the free space was to allow a quicker attainment of equilibrium. The trays were held at a constant temperature and the concentration of CO_2 in the dish was measured by circulating air from the sealed tray through a katharometer and back to the tray. The concentration of oxygen was measured by circulating the air through an oxygen meter. In some instances samples were also withdrawn for determination of the concentration of ethylene.

The advantage of using trays covered with films rather than film bags is that trays provide a rigid system and facilitate handling and sampling.

Observations have been made on the conditions in film bags, but great care is needed in handling nonrigid packages and in taking samples from them.

Several aspects of the use of film packages can be studied in this way and four examples may be given.

First, the effect of variation in the production of CO_2 on the concentration of CO_2 within the container can be studied by using a different number of fruits in the tray.

Second, comparative permeability can be demonstrated by covering trays, each containing the same number of similar fruits, with different films.

Third, the actual permeability of films can be calculated from the rate of respiration (calculated from the rise of CO_2 within a container sealed with a metal sheet) and the equilibrium concentration of CO_2 built up within the sealed tray. The permeability can also be determined by introducing into an empty sealed tray sufficient N_2 to lower the concentration of O_2 to 5% and sufficient CO_2 to give a concentration of 15%: the subsequent increase in concentration of oxygen and decrease in concentration of carbon dioxide is then followed.

Fourth, the effect of imperfections in sealing can be studied by comparing the rise of CO_2 and fall of O_2 in trays sealed with intact and punctured films.

3. Results

(a) *The effect of fruit on the concentration of CO_2 and O_2 within sealed containers*

Fig. 1 shows the changes in the concentrations of CO_2 and O_2 within trays containing 6, 12 and 18 apples and covered with polythene, and also within a tray containing 18 apples and sealed with a thin aluminium sheet. All the trays were held at 15°. It can be seen that within the tray sealed with an aluminium sheet the concentration of CO_2 rose and that of O_2 fell at much the same rate with no equilibrium value being attained, whereas when the trays were sealed with polythene the concentration of CO_2 rose to a value at which it either remained fairly constant or fell off somewhat. As the CO_2 concentration rose that of O_2 fell and continued to do so after the CO_2 concentration had reached a steady value. With polythene the extent of the decrease in the concentration of O_2 may approach twice that of the rise in the concentration of CO_2.

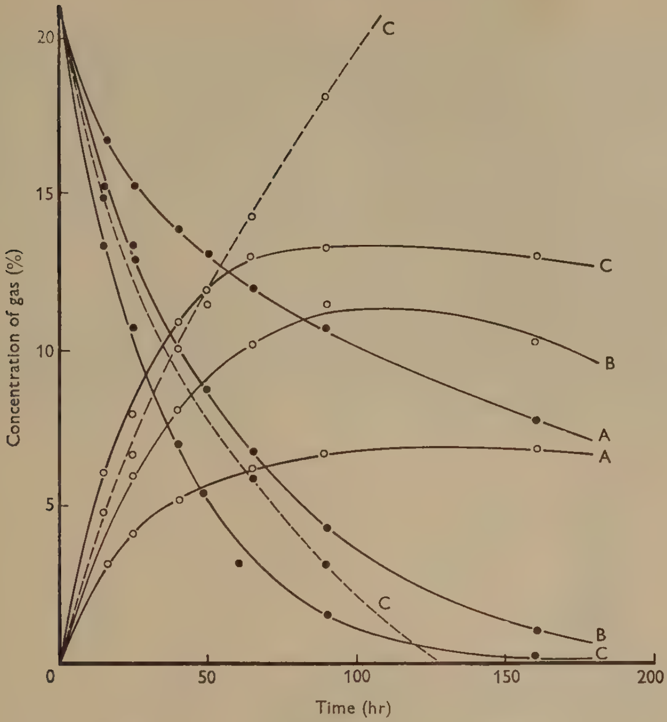


Fig. 1. The effect of type of sealing and number of apples/tray on the changes in the concentrations of CO_2 (open circles) and O_2 (full circles) produced in trays held at 15°. Type of sealing: polythene, continuous lines; metal, broken line. Nos. of apples: A, 6; B, 12; C, 18.

It is clear that the CO_2 concentration rose initially because at first it was produced faster than it could escape through the polythene film, but the amount of CO_2 which escapes increases as its partial pressure rises until the rate of escape is equal to the rate of production and equilibrium is achieved.

Table 1. *The derivation of the permeability of* 150 *gauge polythene film to* CO_2, *using the equilibrium concentrations shown in Fig.* 1

No. of apples/tray	6	12	18
Wt. of apples (g)	562	1,124	1,683
Vol. (ml) of CO_2 produced/day*	182	365	545
Equilibrium concentration of CO_2 (%) (Fig. 1)	6·75	10·5	13·5
Area of film (cm^2)	1,558	1,558	1,558
Permeability of film (ml/cm^2/day)†	0·173	0·230	0·260

* Based on assumed rate of respiration of 135 ml/10 kg/hr.

† Permeability: with a 10% difference between the internal and external concentrations.

From the rise in CO_2 in the tray sealed with metal, one can calculate that the rate of respiration of the apples was 135 ml/10 kg/hr. Assuming that this rate of respiration also obtained in the trays covered with polythene, the rate of diffusion of CO_2 through the polythene film can be calculated from the amounts of CO_2 produced in the tray and the equilibrium concentrations of CO_2 attained, as shown in Fig. 1.

The relevant figures are given in Table 1, from which it appears that an increase in permeability was associated with the larger numbers of apples/tray. This was probably due to a reduction in the rate of respiration as the concentration of CO_2 within the tray rose.

(b) *The effect of punctured sealing films on the changes in the concentrations of CO_2 and O_2 within trays containing fruit*

Fig. 2 shows the changes in concentration of CO_2 and O_2 in trays containing 18 apples and held at 15°, when the polythene film was either intact or had been pierced

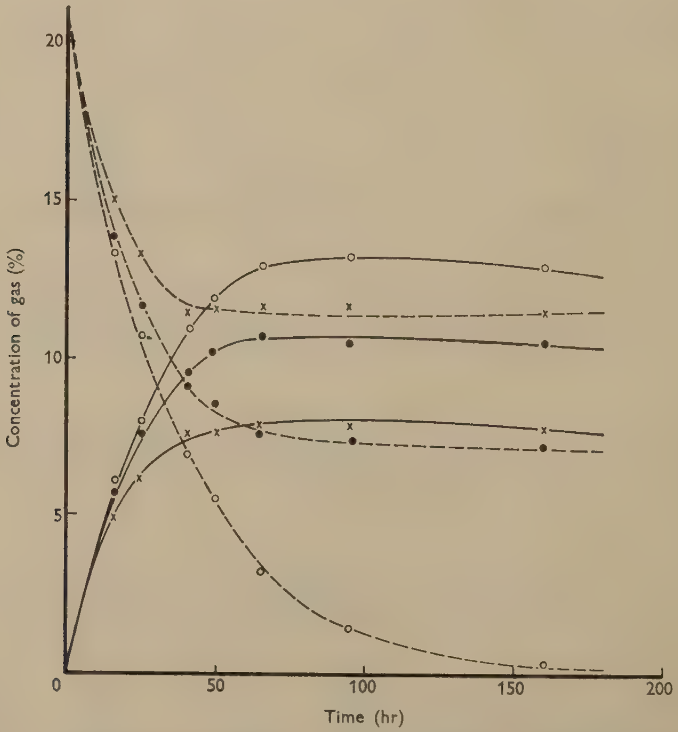


Fig. 2. The effect of intact and punctured polythene sealing films on the changes in CO_2 concentration (continuous lines) and O_2 concentration (broken lines) within trays containing 18 apples and held at 15°. Intact film, open circles; film with 2 punctures, full circles; film with 4 punctures, crosses.

2 or 4 times with a needle. These results were obtained at the same time as those shown in Fig. 1, so that a number of comparisons can be made. It is clear that in the trays with punctured sealing films the increase in concentration of CO_2 was not as great as in the tray with an intact sealing film, and the more holes there were the lower the equilibrium concentration. Further, the fall in O_2 concentration was roughly equal to the rise in that of CO_2 and not greater, as in a tray sealed with an intact film.

The data given in Figs. 1 and 2 can be used to derive the relation between the rise in the CO_2 concentration and the fall in that of O_2, and this is shown in Fig. 3, in which the lines OA and OB (according to the respiratory quotient) represent the relationship for apples in a metal container either sealed or ventilated with holes. In these cases the rise in CO_2 is equal to the fall in O_2. The line OC represents the relationship for intact polythene bags, in which an equilibrium is reached. In these the rise in the concentration of CO_2 is roughly half the fall in that of O_2, because the film is twice as permeable to CO_2 as to O_2.

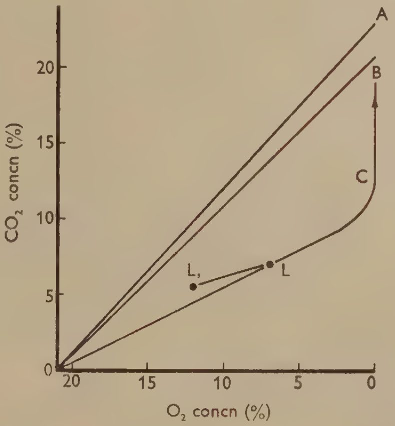


Fig. 3. The relationship between the changes in the concentrations of CO_2 and of O_2 due to the respiration of apples in containers. The lines OA and OB (depending on the respiratory quotient) refer to impermeable (metal) containers either completely closed or ventilated by means of holes. The line OC refers to an intact container made of polythene, which is about twice as permeable to CO_2 as to O_2. The line LL_1 represents the changes following the development of a leak. The conditions in leaky packages must fall between OA and OC, i.e. within the area OAC.

The concentrations of CO_2 and O_2 within punctured polythene bags, in which the gases pass through the holes as well as through the polythene, must lie somewhere between those which are found in an intact bag and those found in an impermeable package ventilated with holes. The fewer and smaller the holes the more nearly will the concentrations approach those represented by the line OC and the more and larger the holes the nearer will the concentrations approach those represented by the line OA; the concentrations must fall somewhere between the two limiting conditions, i.e. in the area OAC. If the figures given in some published papers for the concentrations of O_2 and CO_2 found in packages are looked at in the light of the above it is often easy to decide which bags had leaked and which had not.

(c) *The effect of type of film on the concentrations of CO_2 and O_2 in packages containing fruit*

The equilibrium concentrations of CO_2 and O_2 attained in packages are in part determined by the thickness and nature of the film used. Fig. 4 shows that the build up of CO_2 in polythene packages is roughly proportional to the thickness of the film, and Fig. 5 shows that different materials do not behave alike in their permeability to CO_2 and O_2. As the ratios of the permeabilities to CO_2 and O_2 differ for different materials, the curves in Fig. 5 depend on both the thickness and the type of material. Methods of manufacture also affect permeability and different batches of the same materials of the same thickness often differ in this respect.

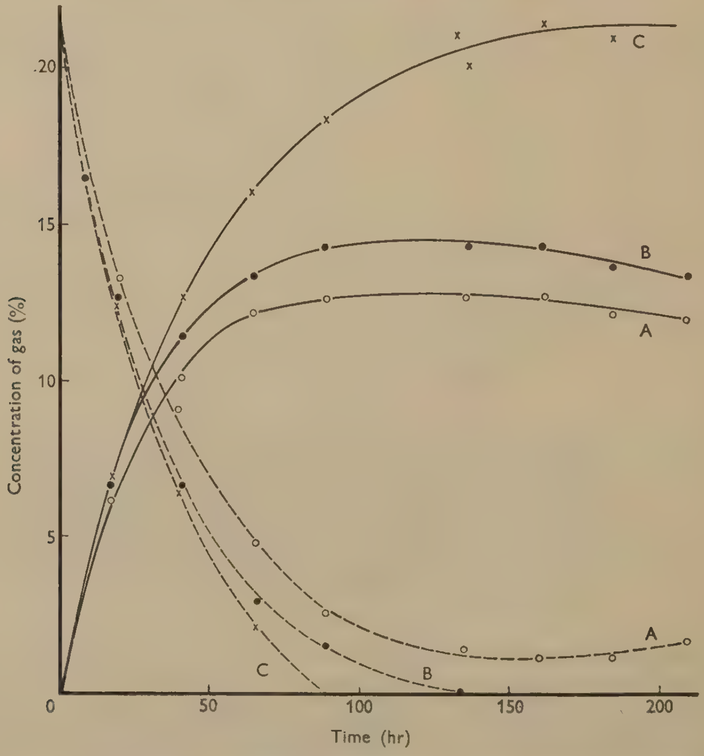


Fig. 4. The effect of thickness of polythene film on the changes in the concentrations of CO_2 (continuous lines) and O_2 (broken lines) in trays containing 9 apples (at 15°). Film thickness (gauge): A, 150; B, 250; C, 500.

The procedure illustrated in Table 1 can be used with data such as those in Fig. 5 to calculate the permeabilities of the various films to CO_2 and O_2. For the purpose of calculating the concentrations of these gases within prepacks of fruits and vegetables it has been found convenient to express permeability in terms of ml of CO_2 or O_2 passing, at 15°, in 1 day through 1 cm^2 of film under a 10% difference between the internal and external concentrations. If for comparative purposes it is further required to correct for thickness of film the choice of a standard of thickness of 0·001 in. (i.e. 100 gauge) is probably most convenient.

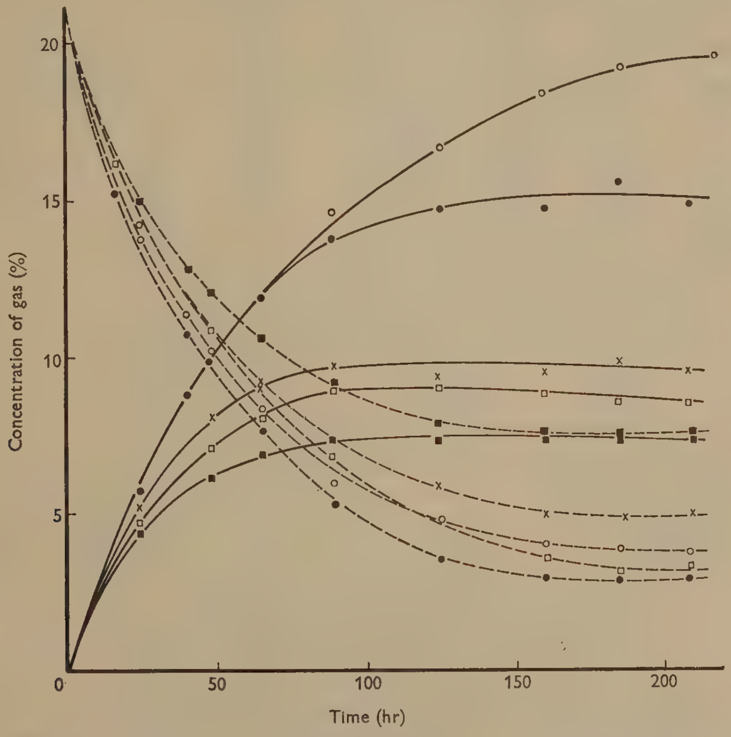


Fig. 5. The effect of the nature of the sealing film on the changes in the concentrations of CO_2 (continuous lines) and O_2 (broken lines) within trays containing 18 apples and held at 15°. Films: cellulose PT, open circles; regenerated cellulose MSAT, full circles; cellulose acetate, crosses; pliofilm, open squares; polystyrene, full squares.

(d) *The accumulation of ethylene in film packages containing apples*

The concentrations of ethylene found in trays of apples sealed with plastic films are given in Tables 2 and 3; they were obtained in the experiments illustrated in Figs. 1, 2 and 5.

In general the changes in the ethylene concentration were similar to those in CO_2, but the relation between the concentrations of CO_2 and ethylene is not direct since the production of the latter is reduced as the concentration of the former increases. No attempt will be made to consider how equilibrium concentrations of ethylene are established in plastic containers; the figures are provided to emphasize that the effects of the accumulation of ethylene on the rate of ripening, and hence on storage life, must be considered in assessing the effects of storage in plastic containers.

Table 2. *The accumulation at 15° of ethylene in trays containing Worcester Pearmain apples and sealed with intact or punctured polythene or intact metal*

Nature of tray seal	No. of apples /tray	Weight of apples (g)	Condition of sealing film	Concn of CO_2 at equilibrium (%)	Concn of ethylene (p/m) after (hr) 25	89	161
Polythene	6	562	Intact	6·75	135	577	372
	12	1,124	Intact	10·5	218	693	780
	18	1,683	Intact	13·5	321	1,033	812
	18	1,679	Punctured	10·5	244	691	578
	18	1,682	Punctured	8·5	215	565	515
Metal	18	1,675	Intact	*	238	930	†

* Equilibrium not established in closed container. † Not determined.

Table 3. *The accumulation at 15° of ethylene in trays containing* 12 *Worcester Pearmain apples and sealed with different materials*

Type of seal	Equilibrium concn of CO_2 (%)	Concn of ethylene (p/m) after (hr) 48	89	126	161	208
Metal sheet	*	512	598	546	565	591
Cellulose acetate	10·0	846	1,011	989	1,006	1,123
Cellulose MSAT 300†	15·0	896	1,032	988	994	1,047
Pliofilm 85	9·0	861	1,101	1,180	1,200	1,302
Polystyrene	7·0	782	787	1,013	1,063	1,195
Cellulose PT‡	17·0	782	911	824	770	755
Polythene 150	8·0	604	674	649	657	755

* Equilibrium not established in closed container.
† MSAT, moisture proof, heat sealing, anchored, transparent.
‡ PT, plain, non-moisture proof, transparent.

It should be mentioned that Worcester Pearmain apples produce more ethylene than most other varieties and that apples produce more ethylene than most other fruits.

(e) *The effect of temperature on the concentrations of CO_2 and O_2 in plastic bags containing fruits*

The rate of respiration of fruits and vegetables increases with temperature, and for comparative purposes it can be assumed to double for every 10° rise. The rate of respiration at T° can also for some purposes be assumed to be roughly proportional to T+5. Hence, if the permeability of a film were not affected by temperature, a rise in temperature of 10° would be expected to double the rate of respiration of CO_2

and therefore double the equilibrium concentration of this gas within the bag. But the permeability of films is influenced by temperature. Brubaker & Kammermeyer (1953) gave figures which indicate that the permeability of polythene to CO_2 and to O_2 increases by 1·55 and 1·7 times, respectively, for every 10° rise in temperature, so that a rise of 10° might well increase the concentration of CO_2 in any bag by 2·0/1·55 or 1·3 times. The relative differences between the rise in the concentration of CO_2 and the fall in concentration of O_2 should also be reduced as the temperature rises. There are data from many sources which show that the concentration of CO_2 in prepacks rises as the temperature is increased, but quantitative relations have not been established. Figs. 6 and 7 show the effect of temperature on the establishment of equilibrium conditions of CO_2 within trays sealed with polythene and pliofilm, respectively. With polythene, the level of temperature within the range 0–15° has an appreciable effect on the concentration of CO_2, but with pliofilm there was in this experiment little effect. The permeability of pliofilm therefore appears to be far more influenced by temperature than that of polythene, but much more experimental work is needed before final conclusions can be drawn about the effects of temperature on the composition of the atmosphere in film packs.

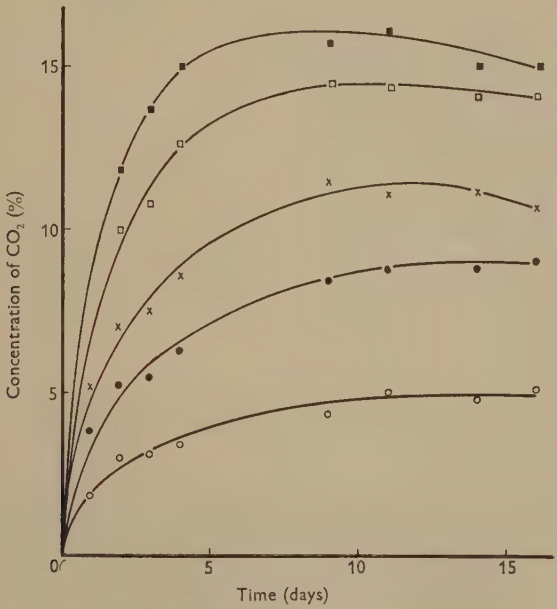


Fig. 6. The effect of temperature of storage on the production of CO_2 within trays containing 6 apples and sealed with 150 gauge polythene. Temperatures: 0°, open circles; 5°, full circles; 10°, crosses; 15°, open squares; 20°, full squares.

Tomkins (1960) gave a graph showing the rates of production of CO_2 by different fruits and vegetables within unit packages, and the critical temperatures at which 10% of CO_2 accumulated. In this graph the rate of escape of CO_2 was also shown, but it was not shown to change with temperature. In Fig. 8 an attempt has been made to show the effect of temperature on both the relative rate of production of CO_2 by three different types of fruit and on the escape of CO_2 from the package. The rate of respiration at 15° is shown to be double that at 5° and the rate of escape of CO_2 at 15° is shown to be double that at –5°. Using this graph it is possible to calculate the equilibrium concentrations of CO_2 within packages at different temperatures, and these are shown in Fig. 9. It is clear that whereas an increase in temperature causes the concentration of CO_2 within a package to rise, the effect of a given temperature increase falls off as the temperature is raised.

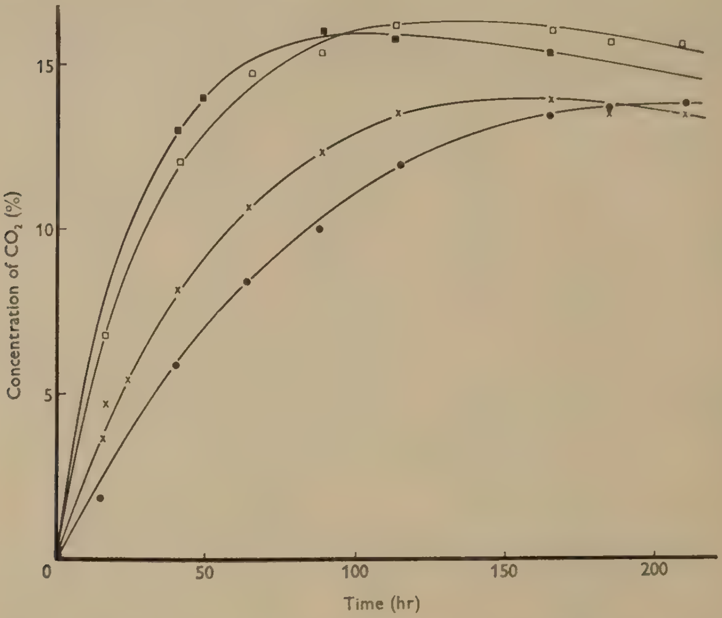
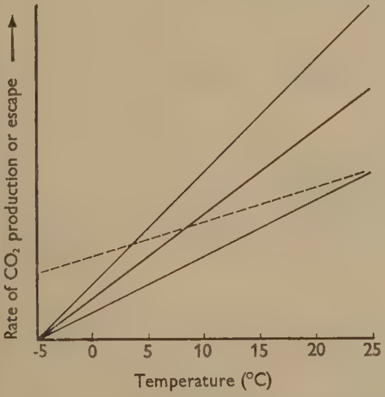


Fig. 7. The effect of temperature of storage on the production of CO_2 within trays containing 18 Worcester Pearmain apples and sealed with 75 gauge pliofilm. Temperatures: 6°, full circles; 10°, crosses; 15°, open squares; 20°, full squares.

(f) *The conditions of humidity in plastic containers*

Fresh fruits and vegetables have water contents of from 80 to 97% and exert vapour pressures of 96 to 98% of that of water at the same temperature. Hence, if enclosed within an impermeable package, they will tend to produce an atmosphere with a relative humidity of 96–98%, and within a plastic container which restricts evaporation the humidity will also tend to increase to that level. However, films differ in their permeability to water vapour. Plain transparent cellulose and cellulose acetate are relatively permeable, whereas polythene is relatively impermeable. But the extent to which a film reduces evaporation depends on the evaporating system, and some figures for the relative extents to which different films affect water losses from different systems are given in Table 4.



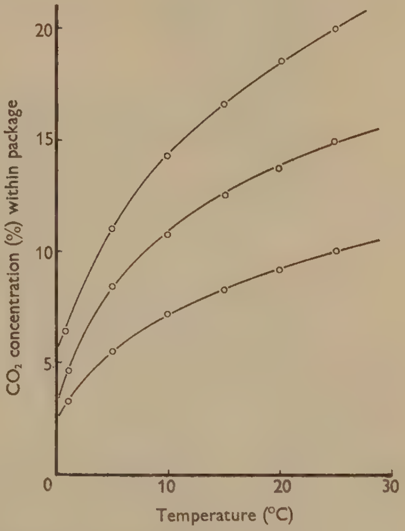


Fig. 8. The effect of temperature on the relative rates of production of CO_2 (continuous lines) by three different fruits sealed in plastic containers, and on the rate of its escape (broken line).

Fig. 9. The effect of temperature on the equilibrium concentrations of CO_2 within film packages containing fruits having different rates of respiration. Data derived from Fig. 8.

Although at constant temperature the relative humidity within packages should not exceed 96–98% (and should be less with a film which is permeable to water vapour), fluctuations of temperature will result in the deposition of films of moisture on the surface of the package and once these are formed saturated conditions are established. The formation of 'fogs' on the inner surface of prepacks of fruits and vegetables is undesirable for several reasons and should be reduced to a minimum by maintaining the temperature as constant as possible. They can also be reduced by making holes in the bag and allowing an increase in water loss. In practice most produce is in fact handled in perforated bags, the number of perforations being adjusted to give the required reduction of water loss. There is, of course, no accumulation of CO_2 in extensively perforated bags.

Table 4. *A comparison of the effects of different sealing films on the relative rates of loss of water at 18° and 70% R.H. from a free water surface and two kinds of fruit*

Type of film seal	Relative loss from system*		
	(a)	(b)	(c)
None	1·0	1·0	1·0
Cellulose PT†	0·15	0·78	0·80
Cellulose acetate	0·09	0·71	0·78
Polystyrene	0·013	NT**	NT
Cellulose QSAT‡	NT	NT	0·68
Pliofilm	0·0127	0·23	0·37
Cellulose MSAT§	0·0042	0·16	0·35
Polythene	0·0042	0·08	0·20
Metal	NT	NT	0·17

* Evaporating systems and their rates of water loss when unprotected: (a), free water surface, 1·0=24 g/cm²/day; (b), Worcester Pearmain apples, 1·0=0·64%/day; (c), tomatoes, 1·0=0·35%/day. ** NT, not tested.

† PT, plain, transparent. ‡ QSAT, moisture permeable, heat sealing, anchored, transparent. § MSAT, moisture proof, heat sealing, anchored, transparent.

(g) *The effects of the conditions of humidity and of the concentrations of CO_2 and O_2 produced in plastic containers on the storage life of the produce*

The storage life of fruits and vegetables can be terminated by excessive evaporation, by over ripening, by the onset of physiological disorders or by rotting or other types of spoilage due to fungi and bacteria.

The use of plastic bags provides a simple means of restricting water loss. Their use can however create conditions of high humidity which lead to increased wastage. If plums are stored at a high humidity their skins crack as they ripen. Apples may also split if they are ripened at a high humidity and they are also more subject to such physiological diseases as superficial scald and low temperature breakdown under such conditions. Contact of fruits with films of water can also lead to splitting. This is followed by the exudation of cell sap, so that films of nutrient solutions are formed which can support a saprophytic flora causing spoilage of a type not normally met with in the handling of fresh fruits and vegetables.

Storage at high humidities may not always increase the liability of sound fruits and vegetables to rotting. Fruits and vegetables are living organisms and the fungi causing rots are parasites; hence factors other than water supply may limit attack. Infection may be influenced by relative humidity. The effect of extent of evaporation on the incidence of rotting of plums sprayed with spores of *Monilia fructigena* is shown in Fig. 10. The rotting of tomatoes is also greatly influenced by humidity. The development of rots arising from latent infections is not, however, primarily affected by the conditions of humidity, but by the state of maturity of the fruit; such rots can develop only when the host is sufficiently ripe. If packaged fruits and vegetables begin to rot the amount of aerial mycelium produced will be greater than with exposed fruit. The escape of juice, and thus the saprophytic flora, may also be increased.

Conditions of excessive humidity can be reduced by having holes in the package and for certain types of produce this may always be advisable. With other types of produce restriction of ventilation and the build up of humidity and CO_2 may be advantageous. Restriction of ventilation is the basis of the 'controlled atmosphere storage' of apples and some other fruits and vegetables. The optimal concentrations of O_2 and CO_2 for a number of varieties of apples are known and some of this

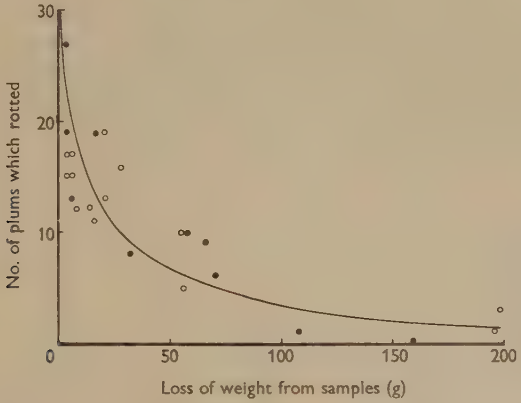


Fig. 10. The effect of extent of water loss at 18° on the numbers of plums out of 40 Reeves Seedlings (wt 1343 g, open circles) and 30 Victorias (wt 1100 g, full circles) which rotted after being sprayed with spores of *Monilia fructigena* and stored in plastic bags punctured with different numbers of holes to allow different degrees of drying.

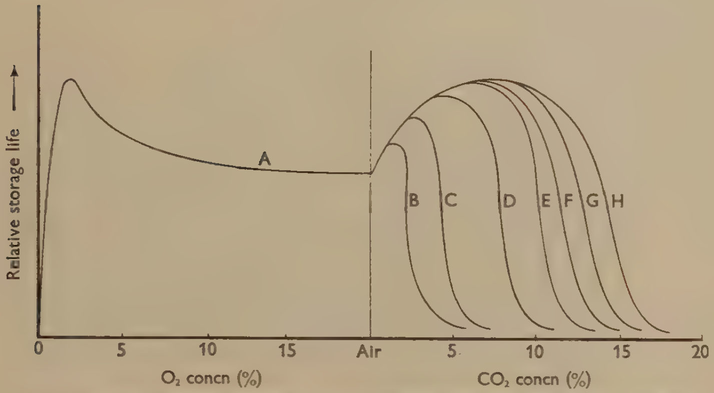


Fig. 11. The effect of the composition of the atmosphere maintained throughout the storage period, on the storage life of apples at 38–40°F, relative to that in air. A, most varieties; B, Newton Wonder; C, Barnack Beauty; D, Cox's Orange Pippin and Ellison's Orange; E, Egremont Russet; F, Worcester Pearmain; G, Laxton's Superb; H, Edward VII and Barney's Seedling.

information is presented in summary form in Fig. 11. Information is also available on the conditions most suitable for other fruits and vegetables.

However, if the concentrations of these gases are not maintained within certain limits damage can be caused, the greatest danger arising from the use of all the O_2 within a package and the establishment of anaerobic conditions. Maintenance of these conditions for only one or two days can result in the formation of 'off' flavours and the death of cells of fruits such as strawberries and raspberries. Apples can in some instances withstand somewhat longer periods without O_2, but exposure of most fruits and vegetables to anaerobic conditions for more than a few days results in serious damage.

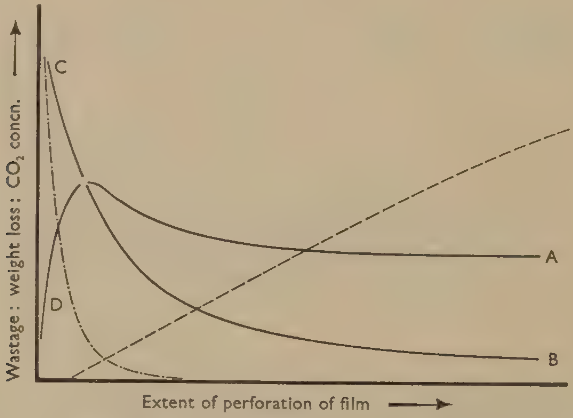


Fig. 12. The possible effects of the extent of perforation of a polythene bag on the accumulation of CO_2 (dash dot line), on weight loss (broken line) and on wastage due to rotting (continuous line). In extensively perforated bags there is no accumulation of CO_2, but appreciable water loss and normal wastage. With fewer perforations weight loss is reduced, the humidity in the bag is increased and CO_2 begins to accumulate. Increased humidity may result in increased rotting. The slight accumulation of CO_2 does not affect rotting. In intact bags water loss is negligible, the humidity high and the CO_2 concentrations appreciable. High humidity may enhance rotting; concentrations of CO_2 above 5% may enhance the rotting of some fruit and retard that of others. A, rotting from well established infection; B, rotting from post harvest infection; C, rotting of type enhanced by high CO_2 concentration; D, rotting of type reduced by high CO_2 concentration.

Most fruits and vegetables can, however, be kept in better condition in an atmosphere containing 3–5% of O_2 than in air. The increase in the concentration of CO_2 can also have a retarding effect on ripening and on the development of rotting. The optimal concentrations of this gas may range from 7–8% for certain varieties of apples to 15–20% for certain soft fruits and 20–30% for blackcurrants. Exposure to concentrations above the optimal may lead to damage, of which 'brown heart' of apples is the best known example. Tomkins (1960) summarized in tabular form some of the data on injury caused by CO_2. Restriction of ventilation also results in the accumulation of ethylene, which at temperatures above 5° can stimulate the ripening of most fruits and cause the yellowing of leaves. Hence, though the maintenance of gas storage conditions within sealed plastic containers is possible, it is not easy and departure from the 'best' conditions could be disastrous.

An attempt to summarize the effects of restricting ventilation by the use of plastic containers is given in Fig. 12. This shows that whereas the conditions produced could prolong the storage life of some fruits, they would decrease that of others. Hence one can say that no attempt should be made to package such fruits as tomatoes or oranges in sealed film packages. Such fruits if prepackaged should be put into perforated bags.

Conditions suitable for some fruits can be attained in packages and the fact that different films have different permeabilities to O_2 and to CO_2 may be used to advantage to attain these conditions. However, in practice it is difficult to be sure that a package is completely sealed. It is clear from many of the published data concerning the conditions in supposedly sealed plastic containers that the containers were 'leaky'. The difficulty of securing non-leaky packages has probably reduced the amount of damage which might otherwise have occurred by decreasing the likelihood of producing anaerobic conditions.

4. Discussion

Hardenburg (1960) recently reviewed the state of the prepacked fruits and vegetables industry in the U.S.A. He gave convenience and hygiene as the most important advantages of this form of marketing, and also made it clear that no attempt should be made to package in sealed containers certain produce such as onions (which may shoot the more readily) and oranges. He did not assess the advantages of sealed packages for other produce, and it should be quite clear from what has been said above that no generalizations can be made. If prepacking of any particular fruit or vegetable is contemplated it is first essential to know how it is likely to react to conditions of restricted ventilation, i.e. to increased concentrations of CO_2 and ethylene and to decreased concentrations of O_2. When this information has been obtained it is possible to decide, from a knowledge of the rate of respiration, the size of package in which at any particular temperature a given concentration of CO_2 is likely to be built up, and to assess roughly the chances of changes of temperature causing excessive concentrations of this gas. It may also be possible to decide that leaky packages—packages made from plastic deliberately provided with pin holes—could give conditions (such as 18% of CO_2 and 3% of O_2) which would be more suitable than those provided by intact packages (such as 10% of CO_2 and 3% of O_2).

Data from straightforward experiments on the effects of prepackaging in which no attempt has been made to discover the conditions within the package should be regarded with suspicion. Though it is quite clear that in some instances film packages can be used to produce desirable gas storage conditions, success can be achieved only in certain limited circumstances.

5. SUMMARY

A method of studying the changes produced in the composition of the air in plastic containers by the respiration of fruits and vegetables, of showing the relative permeability of films and of measuring the permeability of films has been described.

It has been shown that the concentration of CO_2 rises until the pressure is such that the rate of escape by diffusion through the membrane or through holes in the material is equal to the rate of production. The concentration of oxygen meanwhile falls until either the rate of passage of oxygen into the bag is equal to the rate at which it is used up or anaerobic conditions are established.

If the plastic container is intact the fall in the concentration of oxygen is greater than the rise in concentration of carbon dioxide, because most films are more permeable to CO_2 than to O_2. If the container leaks the fall in oxygen is more nearly equal to the rise in CO_2.

An increase in the temperature of storage increases the equilibrium concentration of CO_2 within sealed 'prepacks'.

If ethylene is produced it too increases in concentration within a container.

Plastic containers, by restricting evaporation, create conditions of high humidity around the contents. Changes in temperature can lead to condensation and the formation, within containers, of films of water which create saturated conditions, leach solutes from the produce and support a saprophytic growth of bacteria, yeasts, and moulds.

The effects that changes in the concentrations of CO_2 and O_2, coupled with high humidities, can have on storage life have been considered, together with the possibility of obtaining 'gas storage' or 'controlled atmosphere' conditions within containers.

Some fruits should never be stored in closed plastic film containers. The storage life of other fruits can be improved by storage in sealed plastic film containers and that of others by storage in leaky containers. To produce specific conditions within packages it is necessary to choose packages of the correct size and permeability or 'leakiness' and to hold the temperature within fairly narrow limits.

6. ACKNOWLEDGMENTS

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7. REFERENCES

BRUBAKER, D. W. & KAMMERMEYER, K. (1953). Flow of gases through plastic membranes. *Industr. Engng Chem.* (*Industr.*) **45**, 1148.

GERHARDT, F. (1955). Use of film box liners to extend storage life of pears and apples. *Circ. U.S. Dep. Agric.* no. 965.

HARDENBURG, R. E. (1956). Polythene film box liners for reducing weight losses and shrivelling of Golden Delicious apples in storage. *Proc. Amer. Soc. hort. Sci.* **67**, 82.

HARDENBURG, R. E. (1960). What values accrue from prepackaging fresh produce? *Bull. int. Inst. Refrig.* Annexe 1960–3, 227.

MARCELLIN, P. (1959). L'emploi des emballages de matières plastiques comme adjuvants du froid dans la conservation des fruits. *Int. Congr. Refrig. Copenhagen* **3**, 12.

Marcellin, P. (1961). La conservation des pommes ou de poires en atmosphère controlée au moyen d'emballages de matière plastique. *Bull. int. Inst. Refrig.* Annexe 1961–1, 301.

Nicolaisen-Scupin, L. (1960). Polyäthylen-Verpackung bei der Kaltlagerung. *Die Kalte* no. 9, Sept. 1960, 473; no. 10, Oct. 1960, 552.

Tomkins, R. G. (1960). The biological effects of the conditions produced in sealed plastic containers by prepackaged fresh fruits and vegetables. *Bull. int. Inst. Refrig.* Annexe 1960–3, 233.

Tomkins, R. G. (1961). The changes in the concentrations of carbon dioxide and of oxygen produced within sealed plastic packages by fruits and vegetables. *Bull. int. Inst. Refrig.* Annexe 1961–1, 315.

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THE DEVELOPMENT OF A GOOD ROUND CHARACTER

A SPECULATION ON THE ORIGINS OF THE STAPHYLOCOCCUS

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"Twas brillig, and the slithy toves
Did gyre and gimble in the wabe"

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1. INTRODUCTION

INVESTIGATIONS of the composition of stars, comets and meteorites indicate that, in the prebiological era, the surface of the Earth was covered with an atmosphere in which the major components were methane, ammonia and hydrogen cyanide while other components included water vapour, hydrogen sulphide, acetylene, metallic carbides and nitrides. As the Earth cooled, chemical reactions took place between these components and a vast array of organic compounds were formed. Simulation of these conditions in the laboratory shows that, even within short periods of time, many substances now known as components of living cells are formed and there is reason to believe that the whole chemical complex of protoplasm could arise from such a reactive mixture (Oparin, 1953, 1957). Recent investigations by Oro (1961) and Oro & Kimball (1962) have shown that heating hydrogen cyanide at about 70° in aqueous ammonia for several days gives rise to the formation of glycine, alanine, aspartic acid, polypeptides, purines and a variety of substances known to be bioprecursors of purine. Secondary reactions give rise to ribose and deoxyribose while other investigators have, in the past, shown even wider ranges of biological materials produced from similar reaction mixtures in the presence of metallic catalysts.

2. REPLICATION

One of the characteristics of living matter is the ability to reproduce; a chemical complex, whatever its degree of complication and differentiation, is not a living entity until it possesses the ability to replicate or reproduce its own kind. The simplest form of replication that we can imagine on a chemical basis is the coming together of spatially related structures by hydrogen bonding with consequent re-orientation or reaction followed by breaking of the hydrogen bonds and the liberation of replicates, duplicates or complements. This is the basis of the replication postulated for deoxyribonucleic acid molecules by Watson & Crick (1953). DNA consists of a long chain polymer in which the units are the deoxyribonucleotides of adenine, guanine, cytosine and thymine; the sequence of the nucleotides in the polymer is believed to be of definitive importance in determining the characteristics of living cells. Native DNA (i.e. as it exists in living cells) consists of two polynucleotide chains wound together in the form of a double helix. The helical complex is held together by hydrogen bonds between pairs of bases: adenine of one chain being hydrogen bonded to thymine of the other, and a similar bonding exists between guanine of one and cytosine of the other. Just as adenine of one chain can form hydrogen bonds with thymine of the other, so it can bond with another thymine molecule; each chain can therefore form a complementary chain by reacting with the appropriate nucleotides. If both chains of the original helix form complementary chains in this way, and the newly formed chains in turn form a double helix, then the result will be a replication of the original DNA molecule.

Nucleotides as such do not readily react to produce polynucleotides and DNA replication takes place, in present day living material, as the result of the reaction of nucleotide triphosphates with the DNA template or 'primer' in the presence of an enzyme protein, the DNA polymerase (Bessman, Lehman, Simms & Kornberg, 1958; Kornberg, 1957–1958). DNA is the only type of macromolecule present in living cells which is known to multiply by a process of direct replication. DNA is also the centre of the genic organization of matter and consequently we can look upon it not only as the essential ingredient of life, but as the ultimate precursor of life. The living cell can, in turn, be looked upon as the mechanism necessary for the replication of DNA in present conditions. The production of polydeoxyribonucleotides as the result of chemical reaction in primitive Earth conditions is not difficult to explain in an extrapolation of the work of Oro and his colleagues. Such macromolecules form viscous gels and are highly adsorbent; they would therefore produce centres of chemical reaction in an aqueous phase and one could imagine that replication might on occasion take place. Such accidental replication would not compete very effectively for nucleotide residues with all the other reactions going on, unless the replication obtained an advantage by specific catalysis.

The work of the past year has shown that certain combinations of nucleotides in a DNA polymer display an affinity for certain amino acids, and evidence is accummulating that there is a specificity between nucleotide triplets and amino acids such that a given sequence of nucleotides in a polynucleotide chain will correspond to a given sequence of amino acids in a polypeptide chain. The short term experiments of Oro have shown that conditions that give rise to adenine, ribose, deoxyribose, etc. will also give rise to glycine, alanine, aspartic acid and polypeptides. It follows that when the Earth environment had advanced to the stage of the existence of DNA, polypeptides must also have been produced so that, sooner or later, a coincidence would occur between DNA and polypeptide that would give a self-reproducing nucleoprotein with catalytic advantage and 'life' would have begun.

3. Biosynthesis

Note that, at this stage, 'life' consisted of self-reproducing nucleoprotein gels in the midst of organic and inorganic plenitude. Present day organisms have to go to enormous lengths to synthesize the components necessary for the continuation of the nucleoprotein gel. They have to synthesize the purines, the pyrimidines, the pentoses and the amino acids and, during the last fifteen years, biochemists have worked out the biosynthetic pathways involved. Let us take the amino acid arginine. In *Escherichia coli* and *Neurospora crassa* (and many other cells) the synthesis takes place according to the scheme:

$$\text{X} \rightarrow \text{glutamic acid} \rightarrow \text{ornithine} \rightarrow \text{citrulline} \rightarrow \text{arginine.}$$

The intermediate amino acids ornithine and citrulline do not occur as components of the normal proteins of normal cells (and, possibly, do not possess a nucleotide code in the normal DNA). On the primitive Earth arginine would have been available but, as nucleoprotein replication continued, the supply of arginine within a limited locality would be rapidly exhausted and replication would stop—unless a mechanism was available in some gel or other whereby arginine could be made from a precursor such as citrulline. Where such a mechanism was available—and this would involve another specifically active protein—then that particular piece of gel would have an advantage and would outreproduce other pieces of gel. But the ability to utilize citrulline would have no selective advantage while arginine was available. Likewise the ability to utilize ornithine would have no selective advantage while either arginine or citrulline was available. From this argument, Horowitz (1945) has pointed out that biosynthetic pathways must have developed in reverse order to the biosynthesis they mediate.

As such pathways developed, a snag was bound to arise sooner or later in that some essential metabolite was to possess a greater free energy than its available precursor. Then gel reproduction would stop because it could not provide the energy necessary for the exploitation of available precursors. This, one could imagine, was one of the most difficult of the pre-cellular barriers to reproduction that had to be overcome, especially since the process had to take place at temperatures which would not denature the nucleoprotein. The atmosphere at this stage was almost certainly free from oxygen, since any traces of oxygen left during the cooling process would have been taken up by the oxidation processes of rock formation.

Let us look further at the example of arginine synthesis: for formation of citrulline from ornithine is an energy requiring process and present day organisms utilize the energy liberated by the breakdown of adenosinetriphosphate (ATP) for this purpose —

$$\begin{array}{l} NH_3 + CO_2 + ATP = NH_2.CO.PO_3H_2 + ADP \\ \qquad\qquad\qquad\qquad\qquad\quad \swarrow \\ \qquad\qquad\qquad NH_2.CO.PO_3H_2 + \text{Ornithine} = \text{Citrulline} + H_3PO_4 \end{array}$$

The energy rich bond in the ATP molecule is forged by mobilizing the energy of degradation of sugars such as glucose. The fermentation of glucose to lactic acid by the Embden-Meyerhof pathway mediates the uptake of inorganic phosphate during the oxidation of glyceraldehyde phosphate to glyceric acid phosphate with the net formation of ATP, so that the degradation of glucose can be used to drive the synthesis of citrulline from ornithine (Fig. 1).

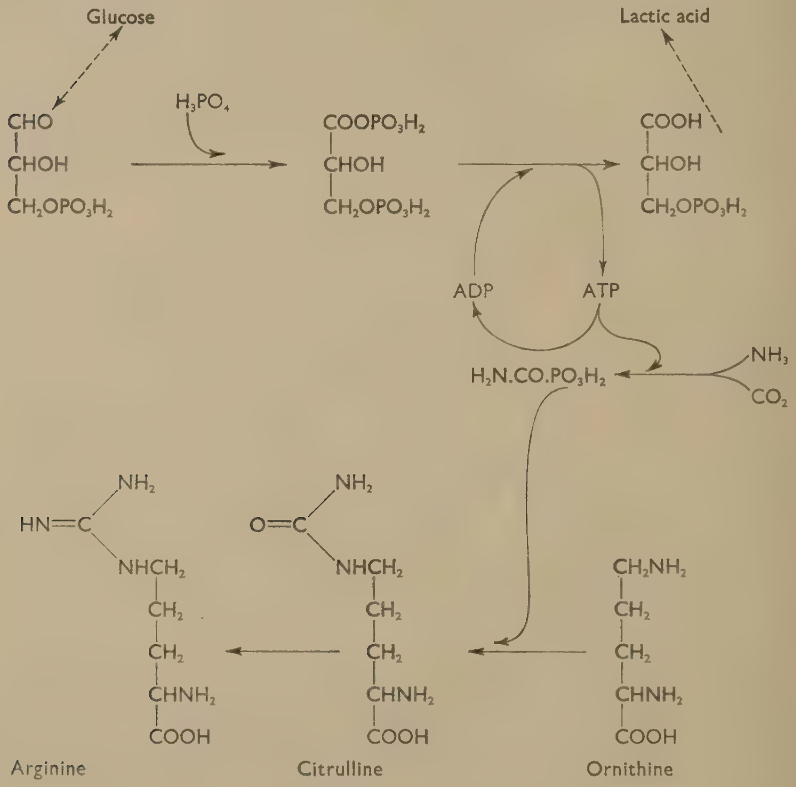

Glucose
Lactic acid
CHO
CHOH
CH₂OPO₃H₂
H₃PO₄
COOPO₃H₂
CHOH
CH₂OPO₃H₂
COOH
CHOH
CH₂OPO₃H₂
ADP
ATP
H₂N.CO.PO₃H₂
NH₃
CO₂
NH₂
HN=C
NHCH₂
CH₂
CH₂
CHNH₂
COOH
NH₂
O=C
NHCH₂
CH₂
CH₂
CHNH₂
COOH
CH₂NH₂
CH₂
CH₂
CHNH₂
COOH
Arginine
Citrulline
Ornithine

Fig. 1. The energy linked synthesis of arginine from ornithine.

The ability to utilize the energy of fermentation in this way requires many additions to our nucleoprotein gel and I am not going to attempt to produce any order or sequence of events in this respect. It is improbable that a single group of coincidences led immediately to the most effective way of burning glucose and we have in the microbial world some extremely diverse ways of dealing with glucose. These ways give us the range of alcohols, aldehydes, ketones, acids, esters and other products which we use for purposes of microbial classification. We must also bear in mind that if the synthesis of arginine requires energy, then the breakdown of arginine will yield energy and if the breakdown is accomplished by a reversal of the reactions set out above, then citrulline breakdown will be linked with the production of ATP. The Gram-positive cocci seem to be unusual among bacteria in that they have retained the ability to use arginine as an energy source for growth by the arginine dihydrolase reaction (Hills, 1940) which involves the formation of ATP in this manner.

It must have been at about this stage in evolution that coincidence occurred between nucleoprotein and a light-absorbing pigment that allowed the utilization of light energy for photosynthesis. From this moment a cataclysmic change took place and life became hard for nucleoproteins. Photosynthesis resulted in the liberation of oxygen. This, in turn, had two far-reaching consequences: most of the chemicals acting as precursors for nucleoprotein reproduction disappeared as a result of oxidation, and oxidative processes offered a more efficient answer to the problem of overcoming energy barriers. The amount of energy to be obtained by the oxidation of glucose can be 20 times that to be obtained by fermentation, so that an entity that could effect oxidation had a big selective advantage over one that was leading an anaerobic existence. The real struggle for existence now began; the ability of a nucleoprotein to survive and reproduce depended upon its ability to exploit its environment, to obtain energy, to manufacture essential materials from simple sources, to avoid destruction and protect that essential reproductive nucleus by every possible means.

4. Evolution of Cellular Existence

The existence of a membrane between the nucleus and the outside world proved a great advantage. This could first have arisen during the process of coazervation when semipermeable monolayers became orientated in the surface of colloidal droplets. In the first place, a semipermeable membrane acts as a sieve. In an aqueous medium the macromolecular nucleoproteins would take up water and a sieve containing quite large holes would be able to prevent the free passage of such micelles. The catalysts—enzymes—that were now essential for biosynthetic purposes are also macromolecules and would equally be held in check and in the same location as the nucleoprotein centre. But what about all the small molecules, the metabolites, that were now necessary as biosynthetic intermediates and were used for the low temperature extraction of energy from fuels? A look at the composition of the membranes of *Staphylococcus aureus* (Mitchell & Moyle, 1956*a*,*b*) and *Micrococcus lysodeikticus* (Gilby, Few & McQuillen, 1958) shows how this problem is dealt with today. The membranes contain 23–28% of lipid and 41–50% of protein; the fatty acid components have been analyzed by Macfarlane (1961, 1962), who found that 70–80% consists of branched chain fatty acids mainly of length 15C. Mitchell & Moyle (1956*b*) pointed out that the composition of the staphylococcal membrane is consistent with a structure containing a monolayer of lipid and a monolayer of protein. Such a structure imposes an effective barrier to the free diffusion of water soluble materials into or out of the cell and so enables the cell to conserve its essential metabolites. On the other hand, there is ample evidence that the protein of the cell membrane is a complex of active molecules concerned in the transport of essential nutrients into the cell across the membrane, which is otherwise impermeable to these substances. The separation of nucleoproteins and enzymes from the external environment by lipoprotein membranes marked the beginning of cellular existence and the biological era.

The chemical potential of the Earth's surface was now very different: organic materials were rapidly exploited by cellular systems and were either incorporated into the cells or degraded to inorganic end products. Cells had therefore to synthesize or parasitize. At one end of the scale were the hardworking autotrophs and photosynthetic organisms, scraping a living from the soil and existing on air; at the other were the capitalists, the blood suckers and the cannibals. In a world of limited energy supply and restricted carbon or nitrogen source, every ability to exploit a peculiarity of the environment was a conspicuous asset. In times of stress, the winners are those who can economize and streamline their production and so we get the emergence of cells which exploited some local condition for energetic gain and cut down on their biochemical complexity.

5. MUTATION, SELECTION AND CONTROL

We must be quite clear about the nature of our statements here and avoid any charge of teleology. Replication of DNA is followed by synthesis of those proteins whose sequences are coded by the DNA. Occasionally a mistake occurs during replication and the composition of some protein, synthesized subsequently, is altered with the result that the properties of the protein change; if it is an enzyme then its enzymic activity may be lost or its temperature sensitivity may change or its affinity for its substrate alter. For any one protein, a mistake of this nature occurs once in every 10^7 to 10^{10} replications; the mistake originates in the DNA and, as such, is repeated in future generations of that mutant. In a large population mistakes of this kind will produce many organisms whose properties display some variation on the theme of those of the original parent. In some cases the mistake will be of such a nature that the mutant is unable to reproduce in its normal environment and, unless it can change to a more suitable environment, the mutant will die out. In other cases, the loss of a characteristic may result in the mutant possessing a slight advantage over wild types in the same environment. Suppose, for example, the organisms are growing in an environment containing arginine and a mutant occurs which has lost the ability to synthesize arginine; the mutant will grow by direct assimilation of arginine and will have a small energetic advantage over wild types which will continue to synthesize arginine from precursors. Over a large number of generations, this advantage will enable the mutant to outgrow the wild type as long as arginine is present; it will now be restricted to a habitat containing arginine but, within that habitat, its specialization will be advantageous.

A short digression is indicated at this point. In a rich environment, wild type organisms may thus be at a disadvantage compared with nutritionally exacting mutants in that they will continue to use energy for the synthesis of essential metabolites already available in the medium. It is rarely the case that, in the course of evolution, organisms should not have developed an ability to overcome such disadvantages and today we find examples of bacteria which, while possessing the ability to synthesize amino acids, nevertheless do not express that ability when growing in an amino acid rich medium. For example, *Escherichia coli* can synthesize arginine from glucose and ammonia but, if arginine is added to the growth medium, the synthesis from glucose is almost completely suppressed. This is brought about in two ways: arginine inhibits certain enzymes involved in the biosynthesis of arginine ('feedback inhibition') and also represses the formation of the enzymes in the growing organisms (Vogel, 1957; Gorini & Maas, 1958; Pardee, 1961). Feedback and repressor mechanisms have not been developed by all organisms to the same extent and both mechanisms appear to have arisen at a late stage in microbial evolution.

In the early biological era, we can picture the existence of organisms with widely diverse synthetic and catabolic activities. Mutants would constantly have arisen in the cell population and specific mutants would adopt particular habitats which provided them with a selective advantage. We have discussed the advantage to be obtained by utilization of complex molecules instead of building them from precursors; another type of advantage would be possessed by those organisms able to attack energy sources with high efficiency. Oparin (1953) and Lwoff (1944) both suggested that the early micro-organisms possessed wide fermentative, degradative and synthetic activities and that specialization proceeded by loss of enzymic function.

If we take glucose fermentation as an example of the development of streamlining of the energy production mechanism, we can compare the products of glucose breakdown by *Clostridium acetobutylicum*, *E. coli*, *Staphylococcus aureus* and *Streptococcus faecalis* (Table 1). Oparin suggested that the primitive organism was something akin to *Cl. acetobutylicum*, which tries almost every possible way to extract energy from glucose. Evolution by loss of function then led to selection of the homolactic fermenters as organisms making the most efficient use of carbohydrate sources. We can demonstrate this type of change by loss of function in modern organisms. If we remove iron from the growth medium, or inactivate iron in the cells by treating them with carbon monoxide, then the variety of products formed from glucose by

Table 1. *The products of glucose fermentation by certain bacteria*

Product	Quantity of product (as % of glucose fermented) formed by			
	Cl. acetobutylicum	*Escherichia coli*	*Staph. aureus*	*Strep. faecalis*
CO_2	54·0	12·4	—	—
H_2	1·5	1·2	—	—
Formate	—	0·8	—	—
Acetate	4·7	16·8	3	—
Ethanol	2·4	16·1	10	—
Lactate	—	44·6	85	98
Butyrate	2·1	—	—	—
Butanol	23·0	—	—	—
Acetone	7·2	—	—	—
Succinate	—	5·0	—	—
Acetoin	3·1	—	—	—

The values set out above are representative results and the relative amounts of products for any organism will vary with strain and with experimental conditions.

Cl. acetobutylicum, *Cl. welchii* or *Aerobacter aerogenes* is replaced by lactic acid as sole product, so that these heterofermenters are converted to homolactic fermenters by simple loss of function (Kubowitz, 1934; Pappenheimer & Shaskan, 1944; Waring & Werkman, 1944). Table 1 would suggest that staphylococci represent advanced organisms in that their fermentation approaches a lactic one (Friedemann, 1938); however, the nature and relative proportions of the products can be markedly influenced by growth conditions and the pH of the reaction medium, so that staphylococci are normally described as 'heterolactic fermenters'. Krebs (1937) showed that *Staph. aureus* and *Staph. albus* dissimilate pyruvic acid under anaerobic conditions according to the equation

$$2CH_3.CO.COOH + H_2O \rightarrow CH_3.CHOH.COOH + CH_3.COOH + CO_2.$$

Fosdick & Rapp (1943) confirmed the production of lactic acid from pyruvic acid under anaerobic conditions and also identified the minor products, responsible for the characteristic odour of the staphylococci, as α-keto-γ-hydroxyvaleric acid ($CH_3.CHOH.CH_2.CO.COOH$) and its aldehyde.

An idea of the degree to which an organism has lost its synthetic functions is given by the range of amino acids and factors which it requires for growth. Strains of staphylococci differ widely in their abilities to synthesize amino acids; 25 strains investigated by Gladstone (1937) required 5–12 amino acids for growth and our own investigations have shown that laboratory cultures may be mixtures of nutritional mutants within a strain. All strains require thiamine and nicotinic acid (Knight, 1937) and Richardson (1936) showed that uracil is an additional factor required for anaerobic growth. Reports vary for the growth requirements of *Micrococcus lysodeikticus*; Wolin & Naylor (1957) reported that the organism required biotin and glutamic acid only in addition to salts and glucose; Wessman, Allen & Werkman (1954) used a synthetic medium containing 16 amino acids, purines, pyrimidines, biotin and 8 other B vitamins, and Grula, Shing-kei Luk & Yung-chieh Cho (1961) found an absolute requirement for biotin, a purine and amino acids

Table 2. *A comparison of the amino acid breakdown abilities of* E. coli *and* Staph. aureus

Amino acid	*E. coli*		*Staph. aureus* Deamination
	Deamination Q_{NH_3}	Decarboxylation Q_{CO_2}	Q_{NH_3}
Glycine	15	—	2–13
Alanine	30	—	12
Leucine	10	—	0
Proline	15	—	6
Lysine	—	320	3
Histidine	5	25	0
Tryptophan	(5)	—	0
Serine	800	—	19
Cysteine	25	—	4–6
Aspartate	350	—	6
Glutamate	30	120	1
Arginine	5	150	20–250

although the nature of the amino acid requirement was variable. Since most strains in existence are said to be derived from Fleming's original isolate, it is probable that cultures again represent nutritional mutants within a strain. The selection of organisms with nutritional requirements of this complexity necessarily restricts those organisms to a rich habitat—in fact, a habitat that will normally be found only in conjunction with living materials and their products such as milk, blood and decaying cells. Even such media will contain limited amounts of amino acids and efficient use of these for synthetic purposes would certainly require that they should not be broken down for use as energy sources, etc. Experience shows that highly exacting organisms such as the Gram-positive cocci produce little ammonia from amino acid media and Table 2 compares the deaminase and decarboxylase activities of *Staph. aureus* with those of *E. coli* as an example of an organism with highly efficient synthetic powers.

6. USES OF A MEMBRANE

The low concentration and haphazard distribution of amino acids in staphylococcal habitats would appear to present a hazard to the efficient growth of that organism; but another mechanism has been developed to reduce this hazard. If an organism is dependent for growth on the presence of an amino acid in the medium, then that amino acid must enter the cell before it can be utilized by the synthetic mechanism of that cell. However, as we have seen, organisms have developed a semipermeable membrane which acts as an osmotic barrier to free diffusion between the internal and external media. Most of the naturally occurring amino acids are unable to diffuse through this barrier. An exception for some organisms is lysine. Under physiological conditions lysine is positively charged, whereas the cell surface is negatively charged; consequently lysine ions are attracted towards the cell surface and we find that they pass through the membrane by diffusion and become concentrated within the cell by a Donnan distribution effect (Gale, 1947; Najjar & Gale, 1950). However, the staphylococcal membrane is impermeable to glutamic acid, aspartic acid, histidine and most of the other essential amino acids (Gale, 1953). Nevertheless, if energy is supplied by means of exergonic metabolism, a process of active transport takes place with the result that these amino acids are taken across the membrane and concentrated within the cell. The internal concentration may be as much as 400–500 times that in the external medium and no loss takes place when the cells are suspended in distilled water. When we originally discovered this ability of bacteria to concentrate free amino acids, we believed that the property was restricted to Gram-positive species and the cocci were the most efficient of those we surveyed (Taylor, 1947). At that time isotopically labelled amino acids were not available and the internal concentration had to be assayed by an enzymic method after the cells had been washed free from external amino acid. We know now that such treatment washes free amino acids out of Gram-negative bacteria but not from Gram-positive cells. Cohen & Rickenberg (1956) demonstrated that *E. coli* can effect a concentration gradient across the cell surface of 450–500 times for an amino acid such as valine but that, if the cells are placed in water or an amino acid free medium, rapid equilibration takes place with the result that the internal amino acid is very readily lost. The difference between Gram-positive and Gram-negative cells in this respect relates to the ease of equilibration across the cell membrane; in staphylococci amino acids concentrated within the cell are not lost even if the cells are suspended in water for several hours. Cohen & Monod (1957) have used the word 'permease' to describe the component of the membrane which is responsible for the active transport of a substance against a concentration gradient, and have produced evidence that permeases are specific proteins.

The action of amino acid permeases is to concentrate amino acids within the cell where they are needed for synthetic purposes. This not only enables the cell to concentrate essential nutrients from a deficient medium but also enables it to raise the internal concentrations of such nutrients, relatively and absolutely, to levels at which the synthetic mechanisms can function efficiently. In limiting conditions—as might be encountered, for instance, in skin secretions—it would seem that this concentrating ability would confer an advantage. If this is so, it should be possible to compare the survival under limiting conditions of an organism with efficient permease mechanisms with that of a mutant which has lost the mechanism. Lubin, Kessel, Budrean & Gross (1960) sought for such mutants of *E. coli* by irradiating amino acid requiring strains and plating out the survivors in high and also in minimal concentrations of the required amino acid. Normal auxotrophs grew equally well under both conditions but 'transport deficient' mutants grew only in the high concentration of amino acid. In this way mutants deficient in the transport of histidine, proline and glycine were obtained. In each case, the transport deficient mutant grew normally at an amino acid concentration of 250 μg/ml, more slowly than the normal auxotroph at 100 μg/ml, and was unable to grow at 10 μg/ml, whereas the auxotroph grew normally. In each case it was confirmed that the transport deficient mutant was unable to concentrate the specific amino acid required, although its ability to concentrate other amino acids was unimpaired. These experiments not only demonstrate the specificity of the transport or permease systems but also demonstrate their selective advantage in conditions of restricted amino acid supply.

7. USES OF A CELL WALL

Staphylococci concentrate nucleotides, purines, pyrimidines, phosphate, phosphate esters and many essential metabolites in addition to amino acids. It would seem that the staphylococcal membrane enables the cell to establish an internal medium that provides its nucleoprotein nucleus with an environment akin to that which existed in the prebiological era. However, this high concentration of small molecules in the free state (*Staph. aureus* can effect an internal concentration of 0·2 M glutamic acid alone) will necessarily result in high internal osmotic pressure. Mitchell & Moyle (1956*b*) determined the osmotic pressure of *Staph. aureus* and found that it lies between 20 and 25 atmospheres. These are very high values and, at first sight, it would seem impossible that a living organism could withstand such a pressure. Mitchell & Moyle calculated that the tension in the surface of an organism of the approximate size of *Staph. aureus* would be about four times the surface tension of water and pointed out that spherical organisms would be capable of withstanding higher pressures than rod shaped ones of the same radius. Actual measurements of the osmotic pressures of typical rod shaped organisms showed that these are of the order of 5–6 atmospheres. In order to prevent cellular explosion, the organism must be able to synthesize a container of material possessing great tensile strength and, for the greatest efficiency, these containers should be spherical. We may note in passing that it is not surprising that the Gram-positive cocci should be remarkably sensitive to the action of antibiotics such as penicillin which impair the synthesis of the outer wall substance.

We must now find out how staphylococci and micrococci contend with this pressure problem. The membrane in these cells is a very delicate structure. Protoplasts instantly lyze on being suspended in water or hypotonic salt solution. This is readily demonstrated by treating suspensions of *M. lysodeikticus* with the enzyme lysozyme which digests away the outer wall and leaves the membrane untouched. If the digestion takes place in dilute buffer solution, complete lysis follows the lysozyme action. If, however, the osmotic pressure of the cell contents is balanced by the addition of sucrose or other nonpenetrating solute to the medium, then the pressure on the membrane is balanced and stable protoplasts emerge. The difference in osmotic stability of the cell and the protoplast is explained by the presence around the former of the cell wall which encases and protects the protoplast membrane as the outer case of a car tyre encases and protects the inner tube.

Research of the last 10 years has provided a wealth of information concerning the chemistry and structure of bacterial cell walls (reviewed by Salton, 1960). The organisms which form the subject of this symposium have been investigated in particular detail since the wall of *M. lysodeikticus* is one of the few which is completely susceptible to digestion by lysozyme, and the studies of the staphylococcal wall provided the key to the mode of action of penicillin. The work stemmed from the observation of Dawson (1949) that shaking suspensions of staphylococci with ballotini in a sonic vibrator resulted in separation of the walls from the cells and gave material from which preparations of walls could be made. The technique was later used by Salton and others to make wall preparations from a wide variety of bacteria and then to study the chemistry and ultrastructure of the wall substance. It was found that there are considerable differences in the chemistry of walls from different organisms and that, in general, the chemical composition is characteristic of the organism from which the wall has been isolated. Walls of Gram-negative organisms contain more lipid and protein than those of Gram-positive organisms, but this may be a reflection of the fact that the membrane and wall substance are more intimately related in the Gram-negative than in the Gram-positive organisms. The major component of the walls of Gram-positive organisms such as staphylococci and *Micrococcus lysodeikticus* is a mucopeptide which, on hydrolysis, gives glucosamine, N-acetylmuramic acid, DL-alanine, D-glutamic acid, lysine or diaminopimelic acid, and glycine (or, in some cases, serine or aspartic acid). A similar mucopeptide is found in Gram-negative walls but forms less than 10% of the preparation from an organism such as *E. coli*. When *Staph. aureus* grows in the presence of an inhibitory concentration of penicillin, uridine diphosphate derivatives of muramic acid peptides accumulate (Park, 1952) and it has been suggested that these are precursors of the wall substance (Park & Strominger, 1957). The most complex of the nucleotide derivatives formed under these conditions contains the peptide sequence L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine (Park, 1952; Strominger, 1962). The cell wall contains glycine in amount corresponding to 4 glycine residues to 1 glutamic acid residue; there may be therefore a further peptide structure consisting of glycyl residues or there may be a polyglycine bridge between mucopeptide chains (Strominger, 1962). The mucopeptide of *M. lysodeikticus* is sensitive to the action of the enzyme lysozyme; investigations of the products released by lysozyme digestion indicate that the bond broken is a β(1→4) link between N-acetyl-muramic acid and glucosamine. Piecing these various findings together, we can build up a picture of the probable structure of the wall (Strominger, 1962):

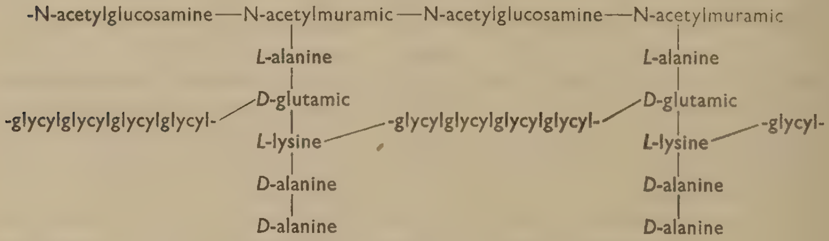

-N-acetylglucosamine—N-acetylmuramic—N-acetylglucosamine—N-acetylmuramic
L-alanine
D-glutamic
L-lysine
D-alanine
D-alanine
-glycylglycylglycylglycyl-
-glycylglycylglycylglycyl-
L-alanine
D-glutamic
L-lysine
D-alanine
D-alanine
-glycyl-

Fig. 2. The possible structure of cell wall mucopeptide.

Mucopeptide forms 95% of the wall of *M. lysodeikticus* (Perkins & Rogers, 1959) and so must be the material which imparts the strength to that structure. Mucopeptide forms some 65% of the wall of *Staph. aureus* and the fact that this organism is not normally sensitive to lysis by lysozyme indicates that its wall must differ in some respects from that of *M. lysodeikticus*. A further series of polymers in the walls of certain Gram-positive organisms (including staphylococci but not *M. lysodeikticus*) has been discovered by Baddiley and his colleagues and named the teichoic acids (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958; Armstrong, Baddiley & Buchanan, 1959; Baddiley, 1962). Two main types of teichoic acid are known: in one the polymerizing unit is ribitol phosphate, in the other glycerol phosphate. Some strains of staphylococci contain ribitol-teichoic acid, some glycerol-teichoic acid, and yet others possess both types in their walls. Both types of teichoic acid possess side chains of amino acids, sugars and amino sugars; the main amino acid is D-alanine and this forms O-ester links with glycerol, ribitol and with the sugars. The ribitol-teichoic acid of *Staph. aureus* also possesses N-acetylglucosamine side chains, while N-acetylgalactosamine has been identified as a component of the glycerol-teichoic acid. The ribitol-teichoic acid of the staphylococcal walls forms the group specific antigen. Strominger (1962) has suggested that D-alanine forms a common link between teichoic acid and mucopeptide in the staphylococcal wall. Since, however, it is possible to remove D-alanine from the teichoic acid without removing the rest of that teichoic acid from the wall (Baddiley, 1962), it is improbable that the two polymers are connected in the way suggested.

Glycerol-teichoic acids of molecular weight smaller than those in the walls are found in the soluble fraction of the staphylococcal cell. These 'intracellular teichoic acids' are precipitated from broken cell preparations along with ribonucleic acid but their function has yet to be elucidated. Cytidine diphosphate glycerol and cytidine diphosphate ribitol have been identified as components of the soluble fraction of the cell, and both substances accumulate when growth is inhibited by penicillin; it is possible that the nucleotides may be concerned in the synthesis of the teichoic acids (Baddiley, Buchanan, Carss, Mathias & Sanderson, 1956; Saukkonen, 1961).

We have built up a picture of the staphylococcal cell as a unit with very high internal pressure, contained within a strong mucopeptide wall. The cell must be contained not only during rest but also during active growth and division; any impairment or attenuation of the wall during the division process would be disastrous. A series of electronmicrographs, prepared by Dr. A. Glauert, of micrococci during division suggests how the cell accomplishes this process (Plate 1). Before any division of the cell has begun, cross septa start to form from points on opposite sides of the cell; these septa grow out across the cell until a complete cross wall has been formed. The cross wall has the appearance of a double wall structure (Plate 1, c) which then splits down the middle as separation takes place, liberating the two daughter cells with the same good, round character as their parent.

8. References

Armstrong, J. J., Baddiley, J., Buchanan, J. G., Carss, B. & Greenberg, G. R. (1958). Isolation and structure of ribitol phosphate derivatives (teichoic acids) from bacterial cell walls. *J. chem. Soc.* 4344.

Armstrong, J. J., Baddiley, J. & Buchanan, J. G. (1959). Teichoic acids from bacterial walls. *Nature, Lond.*, **184**, 247.

Baddiley, J. (1962). Structure and properties of teichoic acids. *Biochem. J.* **82**, 36P.

Baddiley, J., Buchanan, J. G., Carss, B., Mathias, A. P. & Sanderson, A. R. (1956). Isolation of cytidine diphosphate glycerol, cytidine diphosphate ribitol and mannitol 1-phosphate from *Lactobacillus arabinosus*. *Biochem. J.* **64**, 599.

Bessman, M. J., Lehman, I. R., Simms, E. F. & Kornberg, A. (1958). Enzymatic synthesis of deoxyribonucleic acid. II. General properties of the reaction. *J. biol. Chem.* **233**, 171.

Cohen, G. N. & Monod, J. (1957). Bacterial permeases. *Bact. Rev.* **21**, 169.

Cohen, G. N. & Rickenberg, H. V. (1956). Concentration spécifique reversible des aminoacides chez *Escherichia coli*. *Ann. Inst. Pasteur* **91**, 693.

Dawson, I. M. (1949). In the discussion to the paper by Spooner, E. T. C. in *The Nature of the Bacterial Surface*. *Symp. Soc. gen. Microbiol.* no. 1, p. 119. Oxford: Blackwell.

Fosdick, L. S. & Rapp, G. W. (1943). Degradation of glucose by *Staphylococcus albus*. *Arch. Biochem.* **1**, 379.

Friedemann, T. E. (1938). Metabolism of pathogenic bacteria. I. Bacteriological and chemical methods. *J. Bact.* **35**, 527.

Gale, E. F. (1947). The assimilation of amino acids by bacteria. 1. The passage of certain amino acids across the cell wall and their concentration in the internal environment of *Streptococcus faecalis*. *J. gen. Microbiol.* **1**, 53.

Gale, E. F. (1953). The assimilation of amino acids by Gram-positive bacteria and some actions of antibiotics thereon. *Advanc. Protein Chem.* **8**, 285.

Gilby, A. R., Few, A. V. & McQuillen, K. (1958). The chemical composition of the protoplast membrane of *Micrococcus lysodeikticus*. *Biochim. biophys. Acta* **29**, 21.

Gladstone, G. P. (1937). The nutrition of *Staphylococcus aureus*: nitrogen requirements. *Brit. J. exp. Path.* **18**, 322.

Gorini, L. & Maas, W. K. (1958). Feedback control of the formation of biosynthetic enzymes. In *The Chemical Basis of Heredity* (Ed. McElroy, W. D. & Glass, B.), p. 469. Baltimore: Johns Hopkins Press.

Grula, E. A., Shing-Kei Luk & Yung-chieh Cho (1961). Chemically defined medium for growth of *Micrococcus lysodeikticus*. *Canad. J. Microbiol.* **7**, 27.

Hills, G. M. (1940). Ammonia production in pathogens. *Biochem. J.* **34**, 1057.

Horowitz, N. H. (1945). On the evolution of biochemical syntheses. *Proc. nat. Acad. Sci., Wash.*, **31**, 153.

Knight, B. C. J. G. (1937). The nutrition of *Staphylococcus aureus*: nicotinic acid and vitamin B_1; the activity of nicotinamide, aneurin (vitamin B_1) and related compounds. *Biochem. J.* **31**, 731, 966.

Kornberg, A. (1957–1958). Enzymatic synthesis of deoxyribonucleic acid. *Harvey Lect.* **53**, 83.

Krebs, H. A. (1937). Dismutation of pyruvic acid in *Gonococcus* and *Staphylococcus*. *Biochem. J.* **31**, 661.

Kubowitz, K. (1934). Über die Hemmund der Buttersaüre gärung durch Kohlenoxyd. *Biochem. Z.* **274**, 285.

Lubin, M., Kessel, D. H., Budrean, A. & Gross, J. D. (1960). The isolation of bacterial mutants defective in amino acid transport. *Biochim. biophys. Acta* **42**, 535.

Lwoff, A. (1944). *L'Evolution Physiologique*. Paris: Herman et Cie.

MacFarlane, M. G. (1961). Composition of lipid from protoplast membranes and whole cells of *Micrococcus lysodeikticus*. *Biochem. J.* **79**, 4P.

MacFarlane, M. G. (1962). Lipid components of *Staphylococcus aureus* and *Salmonella typhimurium*. *Biochem. J.* **82**, 40P.

Mitchell, P. D. & Moyle, J. (1956*a*). The cytochrome system in the plasma membrane of *Staphylococcus aureus*. *Biochem. J.* **64**, 19P.

Mitchell, P. D. & Moyle, J. (1956*b*). Osmotic function and structure in bacteria. In *Bacterial Anatomy*. *Symp. Soc. gen. Microbiol.* no. 6, p. 150. Cambridge: University Press.

Najjar, V. A. & Gale, E. F. (1950). The assimilation of amino acids by bacteria. 9. The passage of lysine across the cell wall of *Streptococcus faecalis*. *Biochem. J.* **46**, 91.

Oparin, A. I. (1953). *The Origin of Life*. New York: Dover Publ. Inc.

Oparin, A. I. (Editor) (1957). *The Origin of Life on Earth*. Moscow: Publishing House of the Academy of Sciences.

Oro, J. (1961). Comets and the formation of biochemical compounds on the primitive earth. *Nature, Lond.*, **190**, 389.

Oro, J. & Kimball, A. P. (1962). Synthesis of purines under possible primitive Earth conditions. II. Purine intermediates from hydrocyanic acid. *Arch. Biochem. Biophys.* **96**, 293.

Pappenheimer, A. M. & Shaskan, E. (1944). Effect of iron on the carbohydrate metabolism of *Clostridium welchii*. *J. biol. Chem.* **155**, 265.

Pardee, A. B. (1961). Response of enzymic synthesis and activity to environment. In *Microbial Reaction to Environment*. *Symp. Soc. gen. Microbiol.* no. 11, p. 19. Cambridge: University Press.

Park, J. T. (1952). Uridine-5′-pyrophosphate derivatives. I. Isolation from *Staphylococcus aureus*. II. A structure common to three derivatives. III. Amino acid containing derivatives. *J. biol. Chem.* **194**, 877, 885, 897.

Park, J. T. & Strominger, J. L. (1957). Mode of action of penicillin. *Science* **125**, 99.

Perkins, H. R. & Rogers, H. J. (1959). The products of partial acid hydrolysis of the mucopeptide from cell walls of *Micrococcus lysodeikticus*. *Biochem. J.* **72**, 647.

Richardson, G. M. (1936). The nutrition of *Staphylococcus aureus*. Necessity for uracil in anaerobic growth. *Biochem. J.* **30**, 2184.

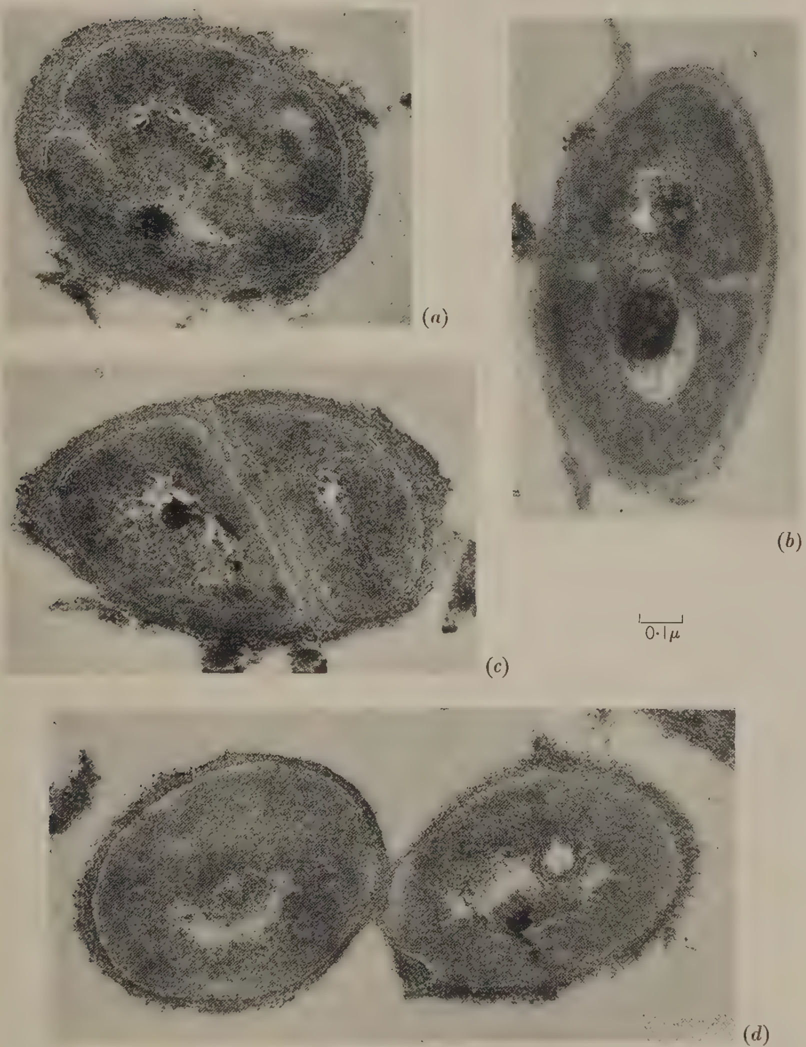
Salton, M. R. J. (1960). *Microbial Cell Walls*. New York: Wiley & Sons.

Saukkonen, J. J. (1961). Acid soluble nucleotides of *Staphylococcus aureus*: massive accumulation of a derivative of cytidine diphosphate in the presence of penicillin. *Nature, Lond.*, **192**, 816.

Strominger, J. L. (1962). Biosynthesis of bacterial cell walls. *Fed. Proc.* **21**, 134.

Taylor, E. S. (1947). The assimilation of amino acids by bacteria. 3. Concentration of free amino acids in the internal environment of various bacteria and yeasts. *J. gen. Microbiol.* **1**, 86.

Journal of Applied Bacteriology, Vol, 25, Part 3



E. F. Gale—The Development of a Good Round Character. A Speculation on the Origins of the Staphylococcus. Plate 1.

VOGEL, H. J. (1957). Repression and induction as control mechanisms of enzyme biosynthesis: The 'adaptive' formation of acetyl-ornithinase. In *The Chemical Basis of Heredity* (ed. MCELROY, W. D. & GLASS, B.), p. 276. Baltimore: Johns Hopkins Press.

WARING, W. S. & WERKMAN, C. H. (1944). Iron deficiency in bacterial metabolism. *Arch. Biochem.* **1**, 303.

WATSON, J. D. & CRICK, F. H. C. (1953). Molecular structure of nucleic acids. *Nature, Lond.*, **171**, 737.

WESSMAN, G. E., ALLEN, L. P. & WERKMAN, C. H. (1954). Biotin requirement of *Micrococcus lysodeikticus*. *J. Bact.* **67**, 554.

WOLIN, L. H. & NAYLOR, H. B. (1957). Basic nutritional requirements of *Micrococcus lysodeikticus*. *J. Bact.* **74**, 163.

EXPLANATION OF PLATE

PLATE 1. Electronmicrographs of four stages in the division of *Micrococcus lysodeikticus* (×67,000). (By courtesy of Dr. A. Glauert, Strangeways Research Laboratory, Cambridge).

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Cowan, S. T. (1962). *J. appl. Bact.* **25** (3), 324–340.

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AN INTRODUCTION TO CHAOS,
OR THE CLASSIFICATION OF MICROCOCCI AND STAPHYLOCOCCI

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1. Introduction

> "The genus *Micrococcus* provides what is probably the worst example in bacteriology of uncritical systematic work." (Abd-el-Malek & Gibson, 1948).

An editor to whom I submitted a paper (Shaw, Stitt & Cowan, 1951) containing this quotation remarked that it was misleading and that the word 'worst' should be changed to 'best'. However we view the classification of micrococci, we must admit that the efforts of systematists have been unfruitful and that after 80 years they have failed to produce a scheme that has a chance of being acceptable even to a bare majority. We need not look far to see chaos in the results, and in Table 1 I have summarized the arrangements made in successive editions of Bergey's *Manual* where, in the sixth edition (Manual, 1948) 17 species were described for the combined staphylococci and micrococci, together with an appendix of names for 28 'species' of staphylococci and 433 additional micrococci.

Table 1. *The numbers of species of micrococci described in Migula* (1900), *seven editions of Bergey's* Manual (1923–57) *and Krassilnikov* (1959)

Genus	Migula (1900)	Bergey's *Manual*, edition							Krassilnikov (1959)
		1 (1923)	2 (1926)	3 (1930)	4 (1934)	5 (1939)	6 (1948)	7 (1957)	
Micrococcus	182	23	23	32	37	44	17*	16	60
Staphylococcus	—	6	5	5	6	6	—	2	—
Sarcina	55	10	10	11	11	11	6	6	18
Rhodococcus	—	5	5	6	6	—	—	—	—
Gaffkya	—	—	—	—	3	3	1	—	—

* Plus an Appendix listing 479 named 'species' of *Micrococcus*, *Staphylococcus*, etc.

I do not pretend to be able to bring order from the micrococcus chaos but I shall try to relate accurately the historical facts (some of which have become distorted), summarize the present position, and look to see where we are going. I shall exclude all strictly anaerobic cocci, which have little except shape in common with the bacteria I shall be dealing with; these are the cluster and packet forming aerobic cocci, catalase positive, and mainly Gram-positive, and I shall call them micrococci, not using the word in a generic sense.

In a generic sense the name *Micrococcus* was first used by Cohn (1872), who wrote: "So as not to encumber the already overburdened synonymy with yet another new word, I have adopted the name *Micrococcus*, established by Hallier and widely known" (translation by H. E. Ross), but according to Buchanan (1925) Hallier's Micrococcus indicated the growth form of a mould and, since he did not use the name in a generic sense, the genus should not be ascribed to him.

Cohn's characterization of *Micrococcus* was, of necessity, vague and mainly based on morphological characters; thus his first species was *Micrococcus prodigiosus*, which we now know to be a short rod and, therefore, excluded from the genus. This is important in nomenclature, for it is often suggested that where the author of a name has not designated a type species the first species mentioned shall be taken as type, and that since Rule 17c of the Bacteriological Code (Code, 1958) says that a name must be changed if the nomenclatural type is excluded, *Micrococcus* is illegitimate. However, the name *Micrococcus* Cohn is saved by Rule 9c which, in subsection 3b, states that "*species inquirendae*, species doubtfully referred to the genus, . . . species which definitely disagree with the generic description (provided others agree), . . . are to be excluded from consideration in selecting the type."

The next species listed by Cohn (1872) was *Micrococcus luteus* (Schroeter) Cohn which, Buchanan (1925) said, ". . . may well be considered the type of the genus as it has been adequately described." Unfortunately, this species was not described as well as Buchanan thought; it has been the subject of a controversy so intense that, when attempts to re-isolate the original organism failed, Evans, Bradford & Niven (1955) proposed that the species be redefined to fit the characterization of a particular strain (ATCC 398) labelled *Micrococcus luteus*.

The isolation of *M. luteus* illustrates the improbability that Cohn ever worked with a pure culture; a needle point of slime was put into a reagent cylinder with a medium containing ammonium tartrate. On the surface a yellow zoogloea formed and multiplied so that a month later there was a thick yellow skin which became folded and crept up the sides of the vessel. Cohn's 1872 paper shows only too clearly that, in most cases at least, he was working with mixed cultures. His characterizations, therefore, are valueless. The genus could be declared illegitimate under Rule 24 which states that "A name of a taxon is illegitimate . . . if the characterization of the group was based upon an impure or mixed culture." This view was taken by Shaw *et al.* (1951) who considered all the Gram-positive, catalase positive cocci to be staphylococci.

Cohn (1872) found the micrococci difficult to classify on the basis of the shape and size of their cells, but he thought that physiological activities were useful; he arranged them into three groups, the chromogenic, zymogenic, and pathogenic cocci. The chromogenic cocci were divided into two subgroups: (i) pigment insoluble—*M. prodigiosus* and *M. luteus*; and (ii) pigment soluble—*M. aurantiacus*, *M. chlorinus*, *M. cyaneus*, and *M. violaceus*. Rather surprisingly Cohn found that a strain produced the same pigment on different media, and he concluded that differences in pigmentation were due to inborn differences or the specific nature of distinct species. The zymogenic cocci were represented by *M. ureae* which, according to Pasteur and others, formed chains and broke down urea to ammonium carbonate. The pyogenic cocci included *M. vaccinae*, the small cocci seen in fresh lymph; *M. diphtericus* (sic), the cocci of diphtheria; *M. septicus*, found in wound secretions, epidemic puerperal fever and tartar from decayed teeth; and *M. bombycis*, a chain forming coccus from silkworm disease.

Ogston (1882) had used the word staphylococcus as a common name, but the generic name *Staphylococcus* should be attributed to Rosenbach (1884), whose name was misspelled Rosenberg by Migula (1900) on p. 135, a mistake that has been copied by other authors (e.g. Krassilnikov, 1959).

At the end of the last century all cocci, whether Gram-positive or Gram-negative, were regarded as members of one family, the Coccaceae. Thus Flügge (1890) put the staphylococci with the streptococci and gonococci in a subdivision 'pathogenic to man' and listed 21 species of saprophytic micrococci.

Migula (1900) divided the Coccaceae into five genera: *Streptococcus*, *Micrococcus*, *Sarcina*, *Planococcus* and *Planosarcina*. *Micrococcus* was subdivided by pigments: white (101 spp. including *M. pyogenes*), yellow (55 spp. including *M. aureus*), red (23 spp.), blue and violet (3 spp.). In addition, there were 19 spp. which would not grow on gelatin and a supplement of 27 insufficiently described species. Fifty-five species of *Sarcina* were listed. But Migula was a realist and knew that many so called micrococci were misplaced; he thought that the unidentifiable species should be regarded as ballast and allowed to slide into eternal oblivion.

The Victorian era was the age of large families in the human race and large genera in bacteriology; Matzuschita (1902) listed 267 spp. of *Micrococcus* in the index to his *Bacteriologische Diagnostik*. Thereafter there was a gradual reduction in the number of species, though not of species names. However, a recent classification by Krassilnikov (1959) recognized 60 spp. of *Micrococcus* and 18 of *Sarcina*.

Classifications of the micrococci have been dominated by attempts to distinguish the pathogens from the saprophytes. In the earlier years of the century the emphasis was on pigmentation, but in the 1930s this changed when the correlation between the coagulase reaction, toxin production and pathogenicity became apparent. It will be convenient to discuss the various proposed classifications under headings indicative of the chief distinguishing character, but it must be admitted that most schemes use several characters.

2. Characters used in Classification

(a) *Pigmentation*

More than any other workers, the Winslows (Winslow & Rogers, 1906; Winslow & Winslow, 1908) gave most weight to pigmentation and divided micrococci into genera, *Aurococcus*, *Albococcus* and *Rhodococcus*, on the basis of the pigment developed by the colony. They also produced an elaborate tinctorial chart by which the colour could be graded. Although Kligler (1913) accepted this arrangement, it was generally agreed (see Walker & Adkinson, 1917) that pigment was insufficient for differentiation, and in 1920 Winslow, Rothberg & Parsons retracted the earlier scheme and substituted one based on sugar reactions, gelatin liquefaction and pigmentation.

Most of those who have attempted to classify micrococci have used pigmentation as one of the distinguishing features, and the well known variation of pigment production by golden staphylococci (Bigger, Boland & O'Meara, 1927; Barber, 1955) is in contrast to the stability of pigment production by the yellow and pink micrococci. The variation is almost always a loss of ability to produce pigment: but by adding an extract from a highly pigmented strain Byatt, Jann & Salle (1948) were able to make a nonpigmented strain develop pigment. It has been suggested (Sevag & Green, 1944) that certain carbohydrates, or some product of their metabolism, are essential for pigment production.

(b) *Biochemical reactions*

At the time when the Winslows were classifying the cocci by pigments, Andrewes & Gordon (1907) were trying to correlate pigmentation and certain biochemical reactions with pathogenicity for guinea-pigs. The strains they examined were of human origin and were divided into four groups. This classification is of historical interest and, because it is not readily accessible, it is reproduced in Table 2. Andrewes & Gordon noticed that the white variants of golden strains had the same biochemical reactions as the parent strains and were just as pathogenic for guinea-pigs. In this connection it is interesting that many years later, when studying dissociates of staphylococci, Doudoroff (1936) found that high haemolysin, high fibrinolysin production and rapid gelatin liquefaction were associated with low pigmentation (i.e. with pale colonies), but coagulase was constant in spite of variation in pigment.

But a biochemical classification of the staphylococci did not find a ready acceptance. Dudgeon (1908) examined 122 strains of human origin in a number of tests including 'sugars' and found almost as many combinations of characters as there were strains; he noted that most strains from pathological sources fermented mannitol. Cummins & Cumming (1913) found 'sugar' reactions unreliable for, on retest 2–3 months later, many of their strains appeared to have changed their fermentative powers. Abd-el-Malek & Gibson (1948) thought that the staphylococci and micrococci formed a linear series.

Table 2. *Andrewes & Gordon's classification of staphylococci* (1907)

Character	Reactions* of			
	S. pyogenes	*S. epidermidis albus*	*S. salivarius*	Scurf staphylococci
Pigmentation	Orange, pale yellow or white	White	White	White
Clot in milk	+	+	−	−
Gelatin liquefaction	+ (rapid)	+ (slow)	−	−
Nitrate reduction	+	+	+	+
Acid from				
Maltose	+	+	+	−
Lactose	+	+	−	−
Glycerol	+	+	+	−
Mannitol	+	−	−	+
Pathogenesis for guinea-pigs	High	Feeble	None	None

* Reactions: +, positive; −, negative.

So far our attention has been directed to the staphylococcus end of the partnership, but in the 1920s Hucker published a series of monographs on the whole group of bacteria we are considering in this Symposium. He (Hucker, 1924*a*) examined 316 cultures of diverse origin and found that of the sugars, glucose, lactose and sucrose gave most information, and that only a few strains produced acid from mannitol. From our later knowledge it seems reasonable to assume that Hucker's strains were mostly from the micrococcus end of the series. He described a curious phenomenon in relation to gelatin liquefaction, which showed two peaks of incidence, most strains becoming positive either at about 14 days or at 90 days. Hucker (1924*b*) put all the strains labelled Micrococcus, Staphylococcus and Rhodococcus into one genus (*Micrococcus*) which he divided into 16 species by pigmentation, nitrate reduction, use of ammonium phosphate and urea as nitrogen sources, gelatin liquefaction, and mass or tetrad formation. In 1928 he added three new species; his serological investigations will be mentioned later. Hucker's ideas have formed the basis of the classification of micrococci in several editions of Bergey's *Manual*.

Julianelle (1922) did not find any correlation between haemolytic activity and biochemical reactions, but in 1937 he rediscovered the apparent association between mannitol fermentation and pathogenicity. Then I (Cowan, 1938) tried to modernize Andrewes & Gordon's 1907 classification in a paper which, by bad presentation, was misinterpreted as a subdivision of the species *S. pyogenes* into three varieties according to the type of haemolysin produced; my intention was to show the correlation between coagulase and the ability to produce one or other toxin (Table 3).

Christie & Keogh (1940) thought that sugars were of little value in classifying staphylococci, but obtained useful information from the coagulase test (properly controlled), pigmentation, and haemolysis on sheep blood agar plates.

Table 3. *Table III of Cowan* (1938) (*simplified*), *showing the correlation of the biological characters of* 93 *gold*, 48 *white and* 5 *yellow staphylococci*

Character*		Nos. of strains (totals in brackets) with the pairs of characters*											
		Acid from mannitol		Haemolysin production				Coagulase formation		V-P reaction		Gelatin liquefaction	
		+ (114)	− (32)	α (87)	α+β (12)	β (4)	− (43)	+ (103)	− (43)	+ (128)	− (18)	+ (131)	− (15)
Colour	gold	89	4	75	12	0	6	87	6	87	6	92	1
	white	22	26	10	0	4	34	14	34	37	11	35	13
	yellow	3	2	2	0	0	3	2	3	4	1	4	1
Acid from mannitol	+			86	12	4	12	102	12	100	14	107	7
	−			1	0	0	31	1	31	28	4	24	8
Haemolysin production	α							87	0	84	3	87	0
	α+β							12	0	12	0	12	0
	β							4	0	0	4	4	0
	−							0	43	32	11	28	15
Coagulase production	+									96	7	103	0
	−									32	11	28	15
V-P reaction	+											119	9
	−											12	6

* Character: +, positive; −, negative.

To Abd-el-Malek & Gibson (1948) must go the credit for introducing a new approach to the classification of the staphylococci and micrococci, and particularly for showing the value of acetoin production as a distinguishing character. They examined 799 strains, mostly from milk, and divided them into three divisions: (1) the staphylococci, which fermented sugars and were relatively sensitive to heat; (2) an intermediate group of obligate aerobes which did not form acid from sugars; and (3) thermoduric dairy micrococci, which produced acid from sugars (the word fermenters was used but by present day convention this should probably be replaced by oxidizers), including *M. luteus* L. & N. Shaw *et al.* (1951) produced a 'lumpers' classification, putting all Gram-positive, catalase positive cocci into one genus, *Staphylococcus*, which they redefined, as all taxonomists do, to suit themselves and to embrace their ideas at that time. Since each division was made on one criterion, this classification pleased the teacher and diagnostician (who could not fail to 'identify' his coccus): but it was a good example of bad taxonomy. However, in nomenclature of the type species, *S. aureus*, it was above reproach (but see later).

Hill (1959) examined the type strains of Shaw *et al.* and a small number of more recently isolated strains, and analyzed his results by methods advocated by Sneath (1957, 1962) and now called numerical taxonomy (Sneath & Sokal, 1962). He concluded that, of the species defined by Shaw *et al.*, only *S. aureus* and *S. roseus* were natural groups and that the other three 'species' were too heterogeneous to be given species rank. Hill suggested that both coagulase positive and coagulase negative, acetoin positive strains (*S. aureus* and *S. saprophyticus*) should be regarded as staphylococci, and that acetoin negative strains (*S. lactis*, *S. roseus* and *S. afermentans*) should be micrococci. Pohja (1960), also using numerical analysis, confirmed Hill's findings and, using additional tests, made further subdivisions which will be mentioned later.

A few years earlier Evans (1947, 1948) had made two significant observations. One was that the correlation between mannitol fermentation and coagulase production was higher when the fermentation test was carried out under anaerobic conditions, which seemed to contradict Cowell's (1939) finding that whereas staphylococci (coagulase reaction not stated) fermented glucose, lactose and sucrose under anaerobic conditions, only 2 of 28 strains fermented mannitol under the same conditions. The other was that when 7·5% of NaCl was added to mannitol agar the acid produced by coagulase positive (but not by coagulase negative) strains was reduced. Evans, Bradford & Niven (1955) used Evans' observations as a basis for separating staphylococci from micrococci on their relation to oxygen in a complex medium containing glucose. They also pointed out that growth in an ammonium salts medium depended on the carry over of essential vitamins and that the test used by Hucker was not suitable for taxonomy. Thatcher & Simon (1957) were not able to confirm the difference in pH value found by Evans *et al.* (1955) when glucose was broken down under aerobic and anaerobic conditions, but it seems possible that differences in technique or the salt concentration of the medium may have been responsible for the discrepant results since Pohja (1960), like Thatcher & Simon, used strains isolated on a medium containing high concentrations of NaCl and found only a slight drop in pH value under anaerobic conditions. However Baird-Parker (1963) has had no difficulty in confirming Evans *et al.* Pohja (1960) subdivided the fermentative strains into the halotolerant (growing on 15% NaCl) and the non-halotolerant. Mossel (1962), however, preferred the anaerobic fermentation of mannitol as the main distinguishing test.

Many workers have tried other tests in attempts to separate either pathogens from nonpathogens or staphylococci from micrococci. There is a tendency among medical workers to regard pathogens as staphylococci and nonpathogens as micrococci, and since a positive coagulase test seems to be the best indicator of potential pathogenicity, for coagulase positive strains to be staphylococci and all coagulase negative strains to be micrococci. This is too extreme a view, but it is one that is prevalent and must be borne in mind when reading medical literature; it is as unrealistic as the classification of these organisms found in Bergey's *Manual* (1948), a classification by Hucker in which coagulase receives only incidental mention.

(c) *Staphylocoagulase*

Since the 1930s, when it was rediscovered, the coagulase reaction has occupied an important place in the characterization and classification of staphylococci. In 1903 Loeb, using goose plasma, reported that a staphylococcus "... has a specific influence in causing coagulation of the blood". Much (1908) confirmed the clotting power of staphylococci for human, horse and sheep plasma and noticed that the clots were dissolved after 3–4 days at 37°, thus demonstrating a fibrinolysin. Much named the clotting property 'staphylokinase'; Lack (1948) and others have done a disservice to bacteriology by ignoring Much's (1908) use of the word staphylokinase and by applying it to staphylococcal fibrinolysin, which Much discovered but did not name, nearly half a century before them. The name staphylocoagulase for the clotting principle we owe to Gratia (1920).

The rediscovery of coagulase, and its association with strains from pathological lesions (and hence presumed pathogenicity), seems to have been made more or less simultaneously in different parts of the world. Papers by Cruickshank (1937) and

Fairbrother (1940) in this country and a series of papers by Chapman and his colleagues (see, for example, Chapman *et al.* 1937, 1938) in the United States of America drew the attention of clinical pathologists to this test for presumptive pathogenicity. Fisher (1936*a*,*b*) studied both the coagulase and fibrinolysin of staphylococci and showed that some strains could destroy fibrinogen before clotting occurred. He also confirmed Walston's (1935) observation that Seitz filtered broth cultures were unsuitable for the test. Although the test is simple, small changes in technique may make all the difference between a positive and negative result, and technique may account for discrepancies in the correlation between coagulase and other characters. Smith (1962), examining strains from dogs, found that slide tests gave fewer positives than tube tests and that human plasma gave fewest positives; rabbit and sheep plasma gave many more. This is the opposite to our findings with human strains, which give far more positives with human and rabbit plasma than with horse, cow or sheep plasma, whereas guinea-pig plasma gives very few positives. Smith (1962) found that strains producing α-lysin were human plasma coagulase positive.

The type of anticoagulant used can affect the result. Some bacteria use citrate and will clot citrated plasma; Lominski & Grossfeld (1948) recommend the addition of heparin to citrated plasma. Many different methods of carrying out coagulase tests have been described and useful papers on technique and comparisons have been published by, among others, Fisk (1940), Chapman, Berens & Stiles (1941), Gillespie (1943) and Williams & Harper (1946). Plasma that has been passed through a Seitz filter is unsuitable for the test. Chapman (1945), who preferred whole human blood to plasma, found that the blood from different individuals varied in its suitability.

Rammelkamp, Hezebicks & Dingle (1950) found that there were three serologically different coagulases, and Barber & Wildy (1958) reported that antisera against coagulases from staphylococci of different phage groups specifically delayed the clotting of plasma by staphylococci of the same phage groups; thus a classification of staphylococci by the coagulases they produce would be similar to a classification by bacteriophage sensitivity.

Coagulase production is a stable character (Blair, 1938) which is retained by L forms (Mattman, Tunstall & Rossmore, 1961). Although it is associated with strains from lesions, it is usually denied that coagulase plays any part in staphylococcal infections (Foster, 1962), and in certain situations (e.g. on heart valves), coagulase negative strains may establish themselves (Cunliffe, Gillam & Williams, 1943).

(d) *Fibrinolysin*

Although coagulase has found a major place in classification, the related property of fibrinolytic activity, first shown by Much (1908), has not received the same attention. Madison (1935) showed that strains from deep lesions in man produced more fibrinolysin (human fibrinogen) than strains from superficial lesions. Animal strains generally do not lyze fibrin clots of human or animal origin (Madison & Dart, 1936; Christie & Wilson, 1941). Fibrinolysin is not produced by strains producing β-haemolysin (Christie & Wilson, 1941; Rountree, 1947).

Neter (1937) found that some staphylococci produced an anticoagulant in broth containing carbohydrate; this was different from fibrinolysin, and he proposed a classification of staphylococci into four groups on the production (or not) of fibrinolysin and anticoagulant.

(e) *Haemolysins*

Only the completely ignorant write of 'haemolytic staphylococci', for haemolytic zones on blood plates may be brought about by a number of different haemolysins and by a lipase (Orcutt & Howe, 1922). Elek & Levy (1954) investigated discrepancies between plate methods and those in which filtrates act on suspended red cells. The α-haemolysin is one expression of α-toxin (Levine, 1939); the other haemolysins, distinct from α-toxin, are known by Greek letters, β (Smith & Price, 1938*a*) acting on sheep and human cells, γ (Smith & Price, 1938*b*) on rabbit cells, and δ acting on sheep, rabbit, human and horse cells (Marks & Vaughan, 1950). Elek & Levy (1950), using a plate method, found that one of the three lysins (α, β or δ) was always produced by coagulase positive strains. Lipase is independent of haemolysins, i.e. α- and β-toxins, and of coagulase (Christie & Graydon, 1941), but Vogelsang, Wormnes & Östervold (1962) found it in 70% of coagulase positive and in only 11% of coagulase negative strains; Davies (1954) found it in a higher proportion (28%) of coagulase negative strains. The α-toxin also destroys rabbit leucocytes (Proom, 1937), but another leucocidin acting on human white cells has been used in a classification of pathogenic staphylococci (Panton & Valentine, 1929, 1932). The α-toxin was found by Selbie & Simon (1952) to be the most important factor in producing lesions in mice following intramuscular inoculation of culture. But mice are generally resistant to infection by cultures of staphylococci, though highly susceptible to intravenous inoculation of α-toxin.

(f) *Other characters*

Laboratory animals are unusually resistant to staphylococcal infection, and tests for pathogenicity have seldom played any part in taxonomic schemes for these organisms. On the other hand, many tests used as indicators of presumptive pathogenicity (e.g. gelatin liquefaction, urea hydrolysis, mannitol fermentation) have been loosely described as 'pathogenicity tests' (Angyal, 1961). Wiley (1961) described a 'virulence test' in which 13-day-old hen embryos were inoculated intravenously.

Among the many tests used in attempts to distinguish the potentially pathogenic strains are phosphatase, lecithinase, and the production of inhibitory substances. For example, Parker (1958) was able to characterize strains responsible for certain superficial lesions (impetigo and pemphigus neonatorum) as penicillin resistant, inhibiting the growth of *Corynebacterium diphtheriae*, egg yolk negative, and able to produce a zone of opacity on horse serum agar. In a similar way Thygeson (1938) used mannitol fermentation as an indicator of conjunctivitis toxin (tested in rabbits).

These and other special tests are likely to play an increasingly important part in distinguishing particular groups (e.g. pathogens) and may ultimately find a place in the general classification of the group. One such test is susceptibility to lysozyme which, in 1935, Thompson & Khorazo found useful in separating sarcina (100% susceptible) from coagulase positive staphylococci (10% susceptible).

3. SEROLOGY AND ANTIGENIC ANALYSIS

Oeding (1960) wrote a comprehensive review of the modern approach to the antigenic structure of staphylococci, but I want to go back to trace the development of serological methods for these organisms. The naive approach of the early serologists has given the impression that serological analysis is simple, reliable and unchanging, whereas in fact the more we learn the more complicated becomes our analysis.

Kolle & Otto (1902), Geisse (1914) and Walker & Adkinson (1917) obtained consistent results by agglutination, and strains from lesions fell into two serological types. Sera against pathogens agglutinated saprophytic strains to low titre (Geisse, 1914) or not at all (Walker & Adkinson, 1917). Hucker (1924*c*), using one strain of each of 15 species, found little overlapping in agglutination tests except between *Micrococcus aureus*, *M. albus*, *M. luteus* and *M. aurantiacus* (which differed from *M. aureus* only in gelatin).

Seedorff (1924) found that complement fixation gave more consistent results than agglutination and distinguished 13 types, of which 6 were represented by only 1 strain each. On the other hand, Hegemann (1937) found that complement fixation gave more nonspecific results than precipitation tests. By using agglutinin absorption Hine (1922) separated two groups, *S. pyogenes* with 3 and *S. epidermidis albus* with 2 types. Mellon & Caldwell (1926) found a multiplicity of antigens shared by *S. aureus* and *S. albus* and thought that both had been derived from a common ancestor.

Using precipitin reactions Dudgeon & Simpson (1928) failed ". . . to show any hard and fast dividing line between staphylococci of different colours or of different cultural reactions", but Julianelle & Wieghard (1935) were more successful and showed two types, A composed of 'apparently virulent strains' and B of 'avirulent' strains. Thompson & Khorazo (1937) found an additional group among the saprophytic strains and Cowan (1938) an additional group among the coagulase positive strains.

Blair & Hallman (1936) did not have success with the precipitin test but recognized 4 groups by cross adsorption tests. Yonemura (1936), using agglutinin absorption, recognized 9 types among 324 strains. A slide agglutination technique using suspensions of heat killed organisms seemed to give more specific reactions, so that 3 types could be recognized (Cowan, 1939); Christie & Keogh (1940) found 6 new types, 4–9, Durfee (1942) added '235 group' and Hobbs (1948) types 10–13. The types 1 to 13 are often referred to as the 13 'international types' (see, for example, Brodie, 1957); this is incorrect as no international body is authorized to grant recognition to staphylococcus serotypes. Although subtypes are recognizable they are not well enough established to be put into the general scheme (Mercier, Pillet & Chabanier, 1950).

Andersen (Andersen, 1943; Andersen & Heilesen, 1951) was the first to apply modern methods of antigenic analysis to staphylococci and found three kinds of antigen: I, heat stable; II, trypsin stable and heat labile; and III, trypsin labile and heat labile. This work formed the foundation of Oeding's (1952) serological analysis and typing scheme for staphylococci, which by 1954 was capable of typing 94·6% of hospital staphylococci (Oeding & Vogelsang, 1954). Its correlation with the phage-typing scheme now in use was published by Oeding & Williams (1958). Strains from animals are usually not typable by these schemes, and White, Rattray & Davidson (1962) found that of 502 strains from bovine udders 94·8% fell into 3 groups (A, B and C) which were quite distinct from the 13 serotypes of the Cowan, Christie & Keogh and Hobbs series.

Yoshida & Hedén (1962) found at least 7 antigenic components in the cell, and 2 in the cell wall of a strain of staphylococcus. By cell wall analysis Cummins & Harris (1956) had shown that the Gram-positive, catalase positive cocci could be divided into 3 groups which did not correspond with subdivisions made on other criteria; their group I contained all the strains regarded as human pathogens or commensals. Chromatographic analysis of bacterial extracts gave different results (Gregory & Mabbitt, 1957) and the grouping corresponded to that of Abd-el-Malek & Gibson (1948).

4. BACTERIOPHAGE-TYPING

Taxonomists can make use of bacteriophage in two ways: by finding out to which phages a bacterium is susceptible, and which phages a bacterium carries. In staphylococcal classification use has been made of the first. The history of phage-typing and the techniques used have been dealt with fully by Wilson & Atkinson (1945), Williams & Rippon (1952) and Anderson & Williams (1956), and Dr. M. T. Parker will speak on the subject in this Symposium. Thus, it only remains for me to discuss the specificity.

The bacteriophages used in the typing scheme act only on coagulase positive strains; thus phage-typing is a method of subdividing one species, *Staphylococcus aureus*: at higher levels it enables us to say that a strain susceptible to one of the typing phages belongs to the species *S. aureus* and not to any other species. There are phages which act on coagulase negative strains, but these seem to be of little or no use in distinguishing species.

5. THE PRESENT AND FUTURE TRENDS

It seems to me that a change occurred in the approach to the micrococcus-staphylococcus problem about the middle of this century. Up till then we had been content with the tools developed by the early bacteriologists at the end of last century. Recently our domain has been invaded by biochemists, geneticists and others who approach our problems with a detached attitude. I would say that the classification proposed by Shaw *et al.* (1951) was the last of the old type bacteriological schemes, and that newer ones, based on more fundamental differences, are likely to gain the acceptance that the old ones were denied. Among the newer schemes we have those of Evans *et al.* (1955) and Baird-Parker (1963), in which a distinction is made between the cocci which ferment glucose anaerobically (staphylococci) and those which attack it only aerobically or do not attack it at all (micrococci); this distinction is confirmed by sensitivity to lysozyme (Baird-Parker, pers. comm.). Other classifications may be based on the nutritional requirements of the cocci.

It is likely, and indeed to be hoped, that the discussions in the Symposium will clarify our views on classification and indicate the direction in which progress can best be made. I will summarize the present position as I see it in the following terms:

1. There is a general wish to be able to distinguish two (possibly three) main groups: *Staphylococcus*, *Micrococcus*, (*Sarcina*).
2. Among staphylococci one species is well defined by the coagulase test; other characters such as pigmentation are of trivial importance.
3. The coagulase negative staphylococci probably all fit into one species.
4. Subdivision of the micrococci is best made into two subgroups: (a) those attacking glucose and other sugars, (b) those not attacking any sugars.
5. Apart from those producing pink colonies, there is no justification for laying stress on pigmentation.
6. The strictly anaerobic cocci should not be included in the genera *Staphylococcus* and *Micrococcus*.
7. Most workers probably disagree with the suggestion of Shaw *et al.* (1951) that the genus *Sarcina* should be limited to the strictly anaerobic cocci. I believe, however, that the reason for making the suggestion (that the first species on which the genus was founded, *Sarcina ventriculi*, was a strict anaerobe) still holds good.

6. NOMENCLATURE

A discussion of the classification of the micrococci would be incomplete without further reference to nomenclature. At the beginning of this review I described how Cohn's *Micrococcus* was not a homogeneous unit, and Gibson (1953) has pointed out that it was not based on a coccus until 1885 when Zopf transferred a species from *Staphylococcus*. Buchanan (1915, 1925) regarded the generic name *Micrococcus* as valid, but in his 1917 paper he recorded the tribe Micrococceae as being generally Gram-negative. If *Micrococcus* is to be recognized, Buchanan (1925) thought that the type species should be *M. luteus*; controversy over the characterization of this species (Abd-el-Malek & Gibson, 1948; Breed, 1952; Gibson, 1953; van Eseltine, 1955; Evans *et al.* 1955; Breed & Hucker, 1957) raged until Dr. Breed died in 1956; since then there has been an armed truce in which Hucker has failed to produce a strain able to grow in Cohn's ammonium tartrate medium.

Another controversy centred on the specific epithet to be used for the type strain of the genus *Staphylococcus*. Breed (1956) maintained that Rosenbach (1884) used *aureus* and *albus* as varietal epithets, the species being *S. pyogenes*; this was countered by Cowan (1955, 1956) who reproduced relevant pages from Rosenbach's book and also asserted that Rosenbach never used as a binomial the combination *Staphylococcus pyogenes*. This argument also was cut short by Dr. Breed's death, and resolved by the Judicial Commission of the International Committee for Bacteriological Nomenclature in Opinion 17 (Editorial Board, 1958) which designated *Staphylococcus aureus* as the type species and NCTC 8532 as the type strain.

The other problem of nomenclature at the generic level concerns the name(s) for the tetrad and packet forming cocci. Should we reserve *Sarcina* Goodsir for the anaerobic cocci, as suggested by Shaw *et al.* (1951), or should we use this name for all packet forming cocci? If *Sarcina* is confined to the anaerobes, the aerobic packet formers could use *Gaffkya*, a name that is being abandoned by modern workers, most of whom fail to recognize *Gaffkya tetragena*.

At the species level nomenclatural argument abounds but cannot even be discussed profitably until the taxonomic problems have been solved and species defined. Meanwhile, for strains which have hitherto undescribed properties, new species names are being coined without adequate reference to the literature and without enough comparative work with previously described species. Thus it seems possible that the biochemically minded bacteriologists, who have been showing us the light and guiding our steps to a more rational taxonomy, may undo all the good they have done and create a new era of nomenclatural chaos.

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8. REFERENCES

ABD-EL-MALEK, Y. & GIBSON, T. (1948). Studies in the bacteriology of milk. II. The staphylococci and micrococci of milk. *J. Dairy Res.* **15**, 249.

ANDERSEN, E. K. (1943). Studies on the specificity of pemphigus staphylococci. *Acta path. microbiol. scand.* **20**, 242.

ANDERSEN, E. K. & HEILESEN, B. (1951). On the occurrence and typing of *Staphylococcus pyogenes* in weeping eczema and the role played by these bacteria in the pathogenesis and course of eczema. *Acta dermato.-venereol., Stockh.*, **31**, 679.

ANDERSON, E. S. & WILLIAMS, R. E. O. (1956). Bacteriophage typing of enteric pathogens and staphylococci and its use in epidemiology. *J. clin. Path.* **9**, 94.

ANDREWES, F. W. & GORDON, M. H. (1907). Report on the biological characters of the staphylococci pathogenic for man. *Rep. med. Offr loc. Govt Bd* 1905–6, App. B, no. 7, 543.

ANGYAL, T. (1961). Studies on the classification, pathogenicity and antigenic structure of staphylococci. I. The occurrence and pathogenicity of Micrococcaceae. *Acta microbiol. Acad. Sci. hung.* **8**, 127.

BAIRD-PARKER, A. C. (1963). A classification of micrococci and staphylococci based on physiological and biochemical tests. *J. gen. Microbiol.* **29** (in press).

BARBER, M. (1955). Pigment production by staphylococci. *J. gen. Microbiol.* **13**, 338.

BARBER, M. & WILDY, P. (1958). A study of the antigenic specificity of staphylococcal coagulase in relation to bacteriophage group. *J. gen. Microbiol.* **18**, 92.

BIGGER, J. W., BOLAND, C. R. & O'MEARA, R. A. Q. (1927). Variant colonies of *Staphylococcus aureus*. *J. Path. Bact.* **30**, 261.

BLAIR, J. E. (1938). The stability of biological and biochemical properties of staphylococci. *J. Bact.* **35**, 52.

BLAIR, J. E. & HALLMAN, F. A. (1936). Serologic grouping of staphylococci. *J. Bact.* **31**, 81.

BREED, R. S. (1952). The type species of the genus *Micrococcus*. *Int. Bull. bact. Nomen. Taxon.* **2**, 85.

BREED, R. S. (1956). *Staphylococcus pyogenes* Rosenbach. *Int. Bull. bact. Nomen. Taxon.* **6**, 35.

BREED, R. S. & HUCKER, G. J. (1957). The status of the genus *Micrococcus* Cohn, 1872. *Int. Bull. bact. Nomen. Taxon.* **7**, 113.

BRODIE, J. (1957). Some observations on the serological typing of *Staphylococcus pyogenes*. *J. clin. Path.* **10**, 215.

BUCHANAN, R. E. (1915). Nomenclature of the Coccaceae. *J. infect. Dis.* **17**, 528.

Buchanan, R. E. (1917). Studies on the nomenclature and classification of the bacteria. IV. Subgroups and genera of the Coccaceae. *J. Bact.* **2**, 603.

Buchanan, R. E. (1925). *General Systematic Bacteriology. History, Nomenclature, Groups of Bacteria.* Baltimore: Williams & Wilkins Company.

Byatt, P. H., Jann, G. J. & Salle, A. J. (1948). Variation in pigment production in *Staphylococcus aureus*. *J. Bact.* **55**, 787.

Chapman, G. H. (1945). Differences in susceptibility of human blood to coagulation by staphylococci. *J. Bact.* **50**, 119.

Chapman, G. H., Berens, C. & Stiles, M. H. (1941). The coagulation of plasma by staphylococci. *J. Bact.* **41**, 431.

Chapman, G. H., Berens, C., Nilson, E. L. & Curcio, L. G. (1938). The differentiation of pathogenic staphylococci from non-pathogenic types. *J. Bact.* **35**, 311.

Chapman, G. H., Lieb, C. W., Berens, C. & Curcio, L. (1937). The isolation of probable pathogenic staphylococci. *J. Bact.* **33**, 533.

Christie, R. & Graydon, J. J. (1941). Observations on staphylococcal haemolysins and staphylococcal lipase. *Aust. J. exp. Biol. med. Sci.* **19**, 9.

Christie, R. & Keogh, E. V. (1940). Physiological and serological characteristics of staphylococci of human origin. *J. Path. Bact.* **51**, 189.

Christie, R. & Wilson, H. (1941). A test of staphylococcal fibrinolysis. *Aust. J. exp. Biol. med. Sci.* **19**, 329.

Code (1958). *International Code of Nomenclature of Bacteria and Viruses*, edited by R. E. Buchanan, S. T. Cowan, T. Wikén & W. A. Clark. Ames, Iowa: State College Press.

Cohn, F. (1872). Untersuchungen über Bacterien. *Beitr. Biol. Pfl.* **1**, heft 2, 127.

Cowan, S. T. (1938). The classification of staphylococci by precipitation and biological reactions. *J. Path. Bact.* **46**, 31.

Cowan, S. T. (1939). Classification of staphylococci by slide agglutination. *J. Path. Bact.* **48**, 169.

Cowan, S. T. (1955). Specific epithet for the type species of *Staphylococcus*. *Int. Bull. bact. Nomen. Taxon.* **5**, 133.

Cowan, S. T. (1956). *Staphylococcus aureus* versus *Staphylococcus pyogenes*. *Int. Bull. bact. Nomen. Taxon.* **6**, 99.

Cowell, E. V. (1939). The relation of aerobiosis to the fermentation of mannitol by staphylococci. *J. Bact.* **37**, 245.

Cruickshank, R. (1937). Staphylocoagulase. *J. Path. Bact.* **45**, 295.

Cummins, C. S. & Harris, H. (1956). The relationships between certain members of the Staphylococcus-Micrococcus group as shown by their cell wall composition. *Int. Bull. bact. Nomen. Taxon.* **6**, 111.

Cummins, S. L. & Cumming, C. C. (1913). A preliminary note on the differentiation of staphylococci. *J. R. Army med. Cps* **20**, 499.

Cunliffe, A. C., Gillam, G. G. & Williams, R. (1943). Bacterial endocarditis associated with a coagulase-negative *Staphylococcus albus*. *Lancet* **ii**, 355.

Davies, M. E. (1954). A study of the diffusible lipase produced by staphylococci and of its immunological activity. *J. gen. Microbiol.* **11**, 37.

Doudoroff, M. (1936). Association of characters among dissociates of *Staphylococcus aureus*. *Proc. Soc. exp. Biol., N.Y.*, **34**, 216.

Dudgeon, L. S. (1908). The differentiation of the staphylococci. *J. Path. Bact.* **12**, 242.

Dudgeon, L. S. & Simpson, J. W. H. (1928). On the differentiation of the staphylococci, with special reference to the precipitin reactions. *J. Hyg., Camb.*, **27**, 160.

Durfee, O. (1942). Classification of 110 strains of *Staphylococcus aureus*. *J. Bact.* **44**, 589.

Editorial Board (1958). Opinion 17. Conservation of the generic name *Staphylococcus* Rosenbach, designation of *Staphylococcus aureus* Rosenbach as the nomenclatural type of the genus *Staphylococcus aureus* (sic) Rosenbach, and designation of a neotype culture of *Staphylococcus aureus* Rosenbach. *Int. Bull. bact. Nomen. Taxon.* **8**, 153.

Elek, S. D. & Levy, E. (1950). Distribution of haemolysins in pathogenic and non-pathogenic staphylococci. *J. Path. Bact.* **62**, 541.

Elek, S. D. & Levy, E. (1954). The nature of discrepancies between haemolysins in culture filtrates and plate haemolysin patterns of staphylococci. *J. Path. Bact.* **68**, 31.

van Eseltine, W. P. (1955). On the inadvisability of separating the genera *Micrococcus* and *Staphylococcus*. *Int. Bull. bact. Nomen. Taxon.* **5**, 53.

Evans, J. B. (1947). Anaerobic fermentation of mannitol by staphylococci. *J. Bact.* **54**, 266.

Evans, J. B. (1948). Studies of staphylococci with special reference to the coagulase-positive types. *J. Bact.* **55**, 793.

Evans, J. B., Bradford, W. L. (Jr.) & Niven, C. F. (Jr.) (1955). Comments concerning the taxonomy of the genera *Micrococcus* and *Staphylococcus*. *Int. Bull. bact. Nomen. Taxon.* **5**, 61.

Fairbrother, R. W. (1940). Coagulase production as a criterion for the classification of the staphylococci. *J. Path. Bact.* **50**, 83.

Fisher, A. M. (1936*a*). The plasma-coagulating properties of staphylococci. *Johns Hopk. Hosp. Bull.* **59**, 393.

Fisher, A. M. (1936*b*). The fibrinolytic properties of staphylococci. *Johns Hopk. Hosp. Bull.* **59**, 415.

Fisk, A. (1940). The technique of the coagulase test for staphylococci. *Brit. J. exp. Path.* **21**, 311.

Flügge, C. (1890). *Micro-organisms, with Special Reference to the Etiology of the Infective Diseases.* London: The New Sydenham Society.

Foster, W. D. (1962). The role of coagulase in the pathogenicity of *Staphylococcus aureus*. *J. Path. Bact.* **83**, 287.

Geisse, A. (1914). Die Differenzierung pathogener und saprophytischer Staphylokokken. *Z. Hyg. InfektKr.* **76**, 282.

Gibson, T. (1953). The status of the generic names *Micrococcus* and *Staphylococcus* and of the species name *Staphylococcus aureus*. Request for an Opinion. File no. 23. *Int. Bull. bact. Nomen. Taxon.* **3**, 129.

Gillespie, E. H. (1943). The routine use of the coagulase test for staphylococci. *Mon. Bull. Ministr. Hlth Lab. Serv.* **2**, 19.

Gratia, A. (1920). Nature et genèse de l'agent coagulant du Staphylocoque ou "Staphylocoagulase". *C. R. Soc. Biol., Paris*, **83**, 584.

Gregory, M. & Mabbitt, L. A. (1957). The differentiation of bacterial species by paper chromatography. V. Preliminary examination of the micrococci. *J. appl. Bact.* **20**, 226.

Hegemann, G. (1937). Untersuchungen über Typendifferenzierung der Staphylokokken. *Zbl. Bakt.* (Abt. 1 Orig.) **140**, 108.

Hill, L. R. (1959). The Adansonian classification of the staphylococci. *J. gen. Microbiol.* **20**, 277.

Hine, T. G. M. (1922). Serological classification of the staphylococci. *Lancet* **ii**, 1380.

Hobbs, B. C. (1948). A study of the serological type differentiation of *Staphylococcus pyogenes*. *J. Hyg., Camb.*, **46**, 222.

Hucker, G. J. (1924*a*). Studies on the Coccaceae. II. A study of the general characters of the micrococci. *Tech. Bull. N.Y. St. agric. Exp. Sta.* no. 100.

Hucker, G. J. (1924*b*). Studies on the Coccaceae. IV. The classification of genus *Micrococcus* Cohn. *Tech. Bull. N.Y. St. agric. Exp. Sta.* no. 102.

Hucker, G. J. (1924*c*). Studies on the Coccaceae. V. Serological studies of the micrococci. *Tech. Bull. N.Y. St. agric. Exp. Sta.* no. 103.

Hucker, G. J. (1928). Studies on the Coccaceae. IX. Further studies on the classification of the micrococci. *Tech. Bull. N.Y. St. agric. Exp. Sta.* no. 135.

Julianelle, L. A. (1922). Studies on haemolytic staphylococci. Haemolytic activity—biochemical reactions—serological reactions. *J. infect. Dis.* **31**, 256.

Julianelle, L. A. (1937). Determination of staphylococcal types by fermentation of mannite. *Proc. Soc. exp. Biol., N.Y.*, **36**, 117.

Julianelle, L. A. & Wieghard, C. W. (1935). The immunological specificity of staphylococci. I. The occurrence of serological types. *J. exp. Med.* **62**, 11.

Kligler, I. J. (1913). A systematic study of the Coccaceae in the collection of the Museum of Natural History. *J. infect. Dis.* **12**, 432.

Kolle, W. & Otto, R. (1902). Die Differencirung der Staphylokokken mittelst der Agglutination. *Z. Hyg. InfectKr.* **41**, 369.

Krassilnikov, N. A. (1959). *Diagnostik der Bakterien und Actinomyceten.* Jena: Gustav Fischer.

Lack, C. (1948). Staphylokinase: an activator of plasma protease. *Nature, Lond.*, **161**, 559.

Levine, B. S. (1939). The unity of the haemolytic, dermonecrotic and lethal properties of staphylococcal exotoxin and of their corresponding counterparts in staphylococcal antitoxin. *J. Path. Bact.* **48**, 291.

Loeb, L. (1903). The influence of certain bacteria on the coagulation of the blood. *J. med. Res.* **10**, 407.

Lominski, I. & Grossfeld, E. (1948). An improved direct coagulase test for the rapid detection of *Staphylococcus aureus*. *Brit. med. J.* **i**, 343.

Madison, R. R. (1935). Fibrinolytic staphylococci. *Proc. Soc. exp. Biol., N.Y.*, **33**, 209.

Madison, R. R. & Dart, E. E. (1936). Veterinary staphylo-fibrinolysin. *Proc. Soc. exp. Biol., N.Y.*, **34**, 299.

MANUAL (1923–57). Bergey's *Manual of Determinative Bacteriology*. Seven editions: 1st, 1923; 2nd, 1926; 3rd, 1930; 4th, 1934; 5th, 1939; 6th, 1948; 7th, 1957. Baltimore: Williams & Wilkins Company.

MARKS, J. & VAUGHAN, A. C. T. (1950). Staphylococcal δ-haemolysin. *J. Path. Bact.* **62**, 597.

MATTMAN, L. H., TUNSTALL, L. H. & ROSSMORE, H. W. (1961). Induction and characteristics of staphylococcal L forms. *Canad. J. Microbiol.* **7**, 705.

MATZUSCHITA, T. (1902). *Bacteriologische Diagnostik*. Jena: Gustav Fischer.

MELLON, R. R. & CALDWELL, D. W. (1926). Studies in microbic heredity. XI. The genetic origin of *Staphylococcus albus* and *aureus* from common ancestral strains. *J. Bact.* **12**, 409.

MERCIER, P., PILLET, J. & CHABANIER, P. (1950). Détermination des types de staphylocoques par l'agglutination. *Ann. Inst. Pasteur* **78**, 457.

MIGULA, W. (1900). *System der Bakterien.* 2. *Specielle Systematik der Bakterien.* Jena: Gustav Fischer.

MOSSEL, D. A. A. (1962). An attempt of classification of the catalase positive cocci (staphylococci and micrococci). *Bact. Proc.* p. 39.

MUCH, H. (1908). Über eine Vorstufe des Fibrinfermentes in Kulturen von Staphylokokkus aureus. *Biochem. Z.* **14**, 143.

NETER, E. (1937). Fibrinolytic, anticoagulating and plasma-clotting properties of staphylococci. *J. Bact.* **34**, 243.

OEDING, P. (1952). Serological typing of staphylococci. *Acta path. microbiol. scand.* Suppl. 93, 356.

OEDING, P. (1960). Antigenic properties of *Staphylococcus aureus*. *Bact. Rev.* **24**, 374.

OEDING, P. & VOGELSANG, Th. M. (1954). Staphylococcal studies in hospital staffs. V. Comparison between serological typing and phage typing. *Acta path. microbiol. scand.* **34**, 47.

OEDING, P. & WILLIAMS, R. E. O. (1958). The type classification of *Staphylococcus aureus*: a comparison of phage-typing with serological typing. *J. Hyg., Camb.*, **56**, 445.

OGSTON, A. (1882). Micrococcus poisoning. *J. Anat., Lond.*, **17**, 24.

ORCUTT, M. L. & HOWE, P. E. (1922). Haemolytic action of a staphylococcus due to a fat-splitting enzyme. *J. exp. Med.* **35**, 409.

PANTON, P. N. & VALENTINE, F. C. O. (1929). Staphylococcal infection and re-infection. *Brit. J. exp. Path.* **10**, 257.

PANTON, P. N. & VALENTINE, F. C. O. (1932). Staphylococcal toxin. *Lancet* **i**, 506.

PARKER, M. T. (1958). Some cultural characteristics of *Staphylococcus aureus* strains from superficial skin infections. *J. Hyg., Camb.*, **56**, 238.

POHJA, M. S. (1960). Micrococci in fermented meat products. Classification and description of 171 different strains. *Suom. Maataloust. Seur. Julk.* no. 96.

PROOM, H. (1937). The inter-relationships of staphylococcal leucocidins. *J. Path. Bact.* **44**, 425.

RAMMELKAMP, C. H. (Jr.), HEZEBICKS, M. M. & DINGLE, J. H. (1950). Specific coagulases of *Staphylococcus aureus*. *J. exp. Med.* **91**, 295.

ROSENBACH, F. J. (1884). *Mikro-Organismen bei den Wund-Infections-Krankheiten des Menschen.* Wiesbaden: J. F. Bergmann.

ROUNTREE, P. M. (1947). Some observations on fibrinolysin and beta toxin of staphylococci. *Aust. J. exp. Biol. med. Sci.* **25**, 359.

SEEDORFF, J. (1924). *Staphylococci—serological and fermentative classification of types. Relation of types as to staphylococ (sic)—sufferings. Vaccine-treatment in cases of acne and furunculoses.* København: Levin & Munksgaard.

SELBIE, F. R. & SIMON, R. D. (1952). Virulence to mice of *Staphylococcus pyogenes*: its measurement and its relation to certain *in vitro* properties. *Brit. J. exp. Path.* **32**, 315.

SEVAG, M. G. & GREEN, M. N. (1944). The role of carbohydrates in the development of pigment by *Staphylococcus aureus*. *J. Bact.* **48**, 496.

SHAW, C., STITT, J. M. & COWAN, S. T. (1951). Staphylococci and their classification. *J. gen. Microbiol.* **5**, 1010.

SMITH, J. E. (1962). Characteristics of staphylococci from the nose of healthy dogs. *J. comp. Path.* **72**, 131.

SMITH, M. L. & PRICE, S. A. (1938*a*). Staphylococcus β-haemolysin. *J. Path. Bact.* **47**, 361.

SMITH, M. L. & PRICE, S. A. (1938*b*). Staphylococcus γ-haemolysin. *J. Path. Bact.* **47**, 379.

SNEATH, P. H. A. (1957). The application of computors to taxonomy. *J. gen. Microbiol.* **17**, 201.

SNEATH, P. H. A. (1962). The construction of taxonomic groups. In *Microbial Classification. Symp. Soc. gen. Microbiol.*, no. 12, p. 289. Cambridge: University Press.

SNEATH, P. H. A. & SOKAL, R. R. (1962). Numerical taxonomy. *Nature, Lond.*, **193**, 855.

Thatcher, F. S. & Simon, W. (1957). Some physiological and toxigenic properties of members of the genus *Micrococcus* in relation to taxonomy. *Int. Bull. bact. Nomen. Taxon.* **7**, 21.

Thompson, R. & Khorazo, D. (1935). Susceptibility to lysozyme of staphylococci. *Proc. Soc. exp. Biol., N.Y.*, **33**, 299.

Thompson, R. & Khorazo, D. (1937). Correlated antigenic and biochemical properties of staphylococci. *J. Bact.* **34**, 69.

Thygeson, P. (1938). Mannitol fermentation as an indicator of conjunctival pathogenicity of staphylococci. *Arch. Ophthal., N.Y.*, **20**, 274.

Vogelsang, Th. M., Wormnes, A. & Östervold, B. (1962). Correlation between staphylococcal phage groups and some staphylococcal enzymes demonstrated by simple methods. *Acta path. microbiol. scand.* **54**, 218.

Walker, I. C. & Adkinson, J. (1917). Study I. Studies on Staphylococcus pyogenes aureus, albus and citreus, and on Micrococcus tetragenus and M. catarrhalis. *J. med. Res.* **35** (new series **30**), 373.

Walston, H. D. (1935). The clotting of plasma through staphylococci and their products. *J. Hyg., Camb.*, **35**, 549.

White, F., Rattray, E. A. S. & Davidson, D. J. G. (1962). Serological typing of coagulase positive staphylococci isolated from the bovine udder. *J. comp. Path.* **72**, 19.

Wiley, B. B. (1961). A new virulence test for *Staphylococcus aureus* and its application to encapsulated strains. *Canad. J. Microbiol.* **7**, 933.

Williams, R. E. O. & Harper, G. J. (1946). Determination of coagulase and alpha-haemolysin production by staphylococci. *Brit. J. exp. Path.* **27**, 72.

Williams, R. E. O. & Rippon, J. E. (1952). Bacteriophage typing of *Staphylococcus aureus*. *J. Hyg., Camb.*, **50**, 320.

Wilson, G. S. & Atkinson, J. D. (1945). Typing of staphylococci by the bacteriophage method. *Lancet* **i**, 647.

Winslow, C.-E. A. & Rogers, A. F. (1906). A statistical study of generic characters in the Coccaceae. *J. infect. Dis.* **3**, 485.

Winslow, C.-E. A., Rothberg, W. & Parsons, E. I. (1920). Notes on the classification of the white and orange staphylococci. *J. Bact.* **5**, 145.

Winslow, C.-E. A. & Winslow, A. R. (1908). *The Systematic Relationships of the Coccaceae.* New York: John Wiley & Sons.

Yonemura, N. (1936). Ueber die immunisatorische Einteilung von *Staphylococcus pyogenes*. *Z. ImmunForsch.* **89**, 392.

Yoshida, A. & Hedén, C.-G. (1962). Some serological properties of staphylococcal cells and cell walls. *J. Immunol.* **88**, 389.

Zopf, W. (1885). *Die Spaltpilze.* 3 Aufl. pp. 51-57. Breslau: E. Trewendt.

(*Received* 18 *July*, 1962)

Pohja, M. S. & Gyllenberg, H. G. (1962). *J. appl. Bact.* **25** (3), 341–351.

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NUMERICAL TAXONOMY OF MICROCOCCI OF FERMENTED MEAT ORIGIN

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1. Introduction

The classification of the micrococci of fermented meat origin has been discussed in a previous paper (Pohja, 1960). It was shown in that paper that the very great majority of these micrococci belong to the *Staphylococcus lactis* group as defined by Shaw, Stitt & Cowan (1951). This group was found to be rather heterogeneous, however, in that three distinct subtypes could be recognized on the basis of salt tolerance and salt requirements for growth, viz. the 'halotolerant', the 'nonhalotolerant' and the 'halophilic' groups. It also seemed that the borderline between certain strains classified as *Staph. saprophyticus* by virtue of their ability to produce acetoin (cf. Shaw *et al.* 1951) and other strains belonging to the 'halotolerant' type of *Staph. lactis* remained quite diffuse. The micrococci met in fermented meat products have thus been the subject of further investigation in order to find out whether a distinct subdivision can be obtained.

In the development of the classification presented by Pohja (1960) methods of numerical taxonomy (cf. Sneath, 1957*a*,*b*, 1962) were also applied. Since the procedure followed by Pohja on some points was different from other current methods of numerical taxonomy it has seemed worth while to make a comparison of the results given by different methods. The original work was based solely on manual operations without the aid of computers. Thus it has also seemed desirable to check the earlier results by the treatment of the original data, or at least some part of them, with an electronic computer.

2. MATERIALS AND METHODS

The pure culture material was the same as in the earlier study (Pohja, 1960) where the material, its origin, isolation and characteristics have been described in detail. From this material (191 strains in total), two different test groups, each containing 62 strains, were selected as indicated in Table 1 (several individual strains were included in both groups).

Table 1. *The composition of the test groups selected for the present study*

Groups or subgroups according to Pohja (1960)		No. of strains in the original material	No. of strains of the present study in test group	
			I	II
Staphylococcus aureus (A)		3	1	0
Staph. saprophyticus (S)		13	5	0
Staph. lactis				
Halotolerant, subgroup	c	34	7	18
	d	85	21	44
	e	6	3	0
	f	4	3	0
	g	5	2	0
Nonhalotolerant, subgroup	i	11	4	0
	k	8	5	0
	l	7	4	0
Halophilic, subgroup	b	3	3	0
Staph. roseus				
Nonhalotolerant, subgroup	h	4	0	0
Halophilic, subgroup	a	2	2	0
Staph. afermentans (N)		6	2	0
Totals		191	62	62

The methods of numerical taxonomy now employed were: the procedure developed by Sneath (1957*b*); the method described by Silvestri *et al.* (1962); and a principle developed by Gyllenberg (manuscript in preparation).

Here it may be emphasized only that the analysis of data was based on calculation of the *similarity index* (S) for each possible pair of strains. This index was computed from the formula $S=100s/(s+d)$, where s is the number of similar positive features (+; +) of the two strains compared and d is the number of different features (+; – or –; +). The S values were employed as such also for the method of Silvestri *et al.* (1962). These authors have computed their 'matching coefficients' by also taking similar *negative* features (–; –) into consideration. The method of Gyllenberg is based on calculation of the highest number of disparities (features which do not match) which can be allowed within the range of statistically probable similarity when two strains from a given material are compared at random. When the number of disparities remains in the permissible range, similarity is supposed.

As regards the terminology, we have adopted the term 'cluster' (Sneath, 1962) for the primary groups produced by numerical methods. The term 'node' is used, as by Silvestri *et al.* (1962), to mean the most central strain of a cluster.

The computer used throughout the work was a Wegematic 1,000 (Computer Centre of the Department of Nuclear Physics at the University of Helsinki). The computer routines were developed by one of the present authors (H.G.G.).

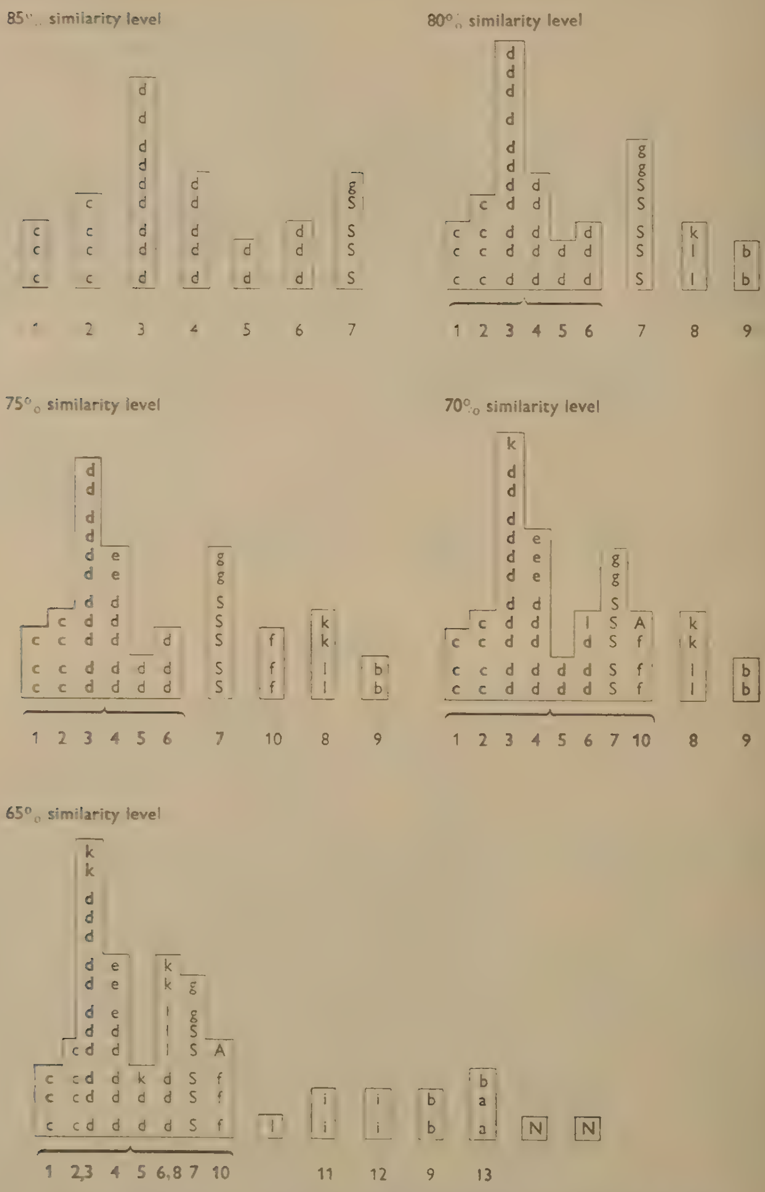
The present report also deals with the development of an identification routine for micrococci of meat origin. For this purpose we used a procedure which, so far as is known to the authors, has not been employed before. The method will be discussed in full elsewhere. Here it may suffice to note that the different tests employed to characterize the isolates are ranked in decreasing order of effect in separating the clusters recognized. To obtain this rank one must know: the degree of intragroup variation as regards each feature (small variation makes the corresponding test more useful); the degree of intergroup separation obtained by a given test—an equal distribution of + and −, respectively, as group characteristics gives best separation; the degree of association of a given feature (evaluated on the basis of group characteristics) to the other features—as unassociated tests are to be included in the set of tests selected for the final identification scheme, a low association figure is beneficial.

From numerical expressions of the above information, a 'rank figure' is then derived for each test, and ranking can be carried out. The tests required for the identification procedure may be selected in the order indicated by the rank.

3. RESULTS

(a) *General survey of 'meat micrococci'*

As can be seen from Table 1, our test group I included representatives of almost all of the types present in the original material of Pohja. The results of the treatment of this test group according to Sneath (1957*b*) is illustrated in Fig. 1, from which the following conclusions can be drawn. First, the halophiles (Pohja's subgroups a and b) were distinctly separable from the other strains, but did not form a uniform group of their own. Accordingly, the occurrence of more than one type of halophilic micrococcus can be suggested. The material studied was limited, however, and does not suffice for a definition of the halophilic groups recognized. In fermented meat products halophilic micrococci seem to be rare. Second, the nonhalotolerant types of *Staph. lactis* (Pohja's subgroups i, k and l) constitute a heterogeneous entirety. The only well definable cluster is that labelled no. 8 in Fig. 1. Other strains (subgroup i) show no relationship to this cluster, and only very low similarity, if any, to each other. Even other strains show high similarity to the large halotolerant group, and may thus represent varieties of this group which have lost their original halotolerance. As a summary of these facts it can be considered that only one distinct nonhalotolerant group is recognizable in the present material, and that representatives of this group do not occur abundantly in fermented meat products. Third, the strains identified as *Staph. saprophyticus* according to Shaw *et al.* (1961) cannot be distinguished from highly



85% similarity level

80% similarity level

75% similarity level

70% similarity level

65% similarity level

1 2 3 4 5 6 7
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 10 8 9
1 2 3 4 5 6 7 10 8 9
1 2,3 4 5 6,8 7 10 11 12 9 13
N
N

Fig. 1. The production and fusion of clusters at different similarity levels as revealed by the procedure of Sneath (1957*b*) applied to test group I, the composition of which is shown in Table 1.

similar acetoin negative strains (Pohja's subgroup g). These strains (*Staph. saprophyticus* plus subgroup g) produce, however, a distinct group of their own which fuses with the bulk of the halotolerant group at the 70% similarity level. Acetoin production obviously cannot be selected as a distinctive characteristic of this group. Fourth, *Staph. aureus* seems to be rather rare in fermented meat products. Pohja's original material included only one strain (out of 171) from this source. This strain showed some relationship (combination at the 70% similarity level) to the halotolerant types, but on the basis of observations concerning this one strain conclusions, of course, cannot be drawn. Fifth, the nonfermentative strains included in test group I showed no mutual relationship, neither were they related to any cluster of other strains recognized. Lastly, the facts referred to above may show that organisms of the halotolerant group of *Staph. lactis* described by Pohja (1960). subgroups c, d, e, f and g, constitute the characteristic micrococcal population of fermented meat products. This group has therefore been subjected to our further study of the taxonomy of the micrococci in question.

(b) *Numerical taxonomy of the halotolerant group of* Staphylococcus lactis

The halotolerant group was defined to include (cf. Pohja, 1960) all strains characterized by the ability to grow both in the presence of 0 and 15% concentrations of NaCl. and moreover by the lack of coagulase, which characteristic excluded all types of *Staph. aureus*. As defined in this way the group included *Staph. saprophyticus* and the acetoin negative strains related to it. This was considered convenient, as it would give further information concerning the relationship of the latter organisms to the bulk of the halotolerant group. The procedure of cluster analysis originally followed by Pohja (1960) shows great analogy to the unweighted variable group method of Sokal & Michener (1958; cited by Sneath, 1962). The report of Sokal & Michener was not known to the present authors, however, at the time when the earlier work was carried out. Pohja's procedure was based on a simultaneous building up of several clusters, starting from cluster nuclei given by pairs of strains showing high similarity. The remaining strains were then investigated one by one and each strain was added to the particular cluster with whose already existing members it produced the highest mean *S* value. This method of analysis resulted in the subdivision of the halotolerant group presented in Table 2.

By treating the test group I according to Sneath (1957*b*) most of Pohja's clusters were easily re-identified (cf. Table 3). A corresponding treatment of test group II. selected to represent the bulk of the halotolerant group of isolates, gave similar results, though it did indicate a further breaking up of the cluster no. 3 produced in the treatment of test group I.

Application of the method of Silvestri *et al.* (1962) gave results in good agreement with Pohja's original data as well as with the picture obtained by Sneath's method. As can be seen from Table 3, where the results of the different procedures have been summarized, the original clusters IIA, IIB, IIC and IID of Pohja were all recognized even with this method. whereas application of the method of Sneath failed to separate

Table 2. *Clusters of halotolerant micrococci of fermented meat recognized in the original study of Pohja* (1960)

Subgroups of halotolerant micrococci	Clusters
c	IA, IB, IC
d	IIA, IIB, IIC, IID, IIE, IIF, IIG, IIH, IX
e	IV
f	VIII
g	III
Staph. saprophyticus	III

Pohja's clusters IB and IC, as well as IIE and IIG, respectively. The procedure of Silvestri *et al.* also indicated that each cluster contains a very homogeneous nucleus of strains just around the node, but also several aggregates of more or less distant strains.

Finally, the method of Gyllenberg failed to separate the original clusters IA, IB and IC, respectively, but separated the clusters IIE and IIG.

On the basis of the results presented in Table 3 the following clusters of halotolerant micrococci were selected for further study (the original cluster labels of Pohja are employed): IA, IBC, IIA, IIB, IIC, IID, IIEG, IIF, IIH, III, IV, and VIII. The strains of *Staph. saprophyticus* included in the pure culture material belong to cluster III.

Table 3. *A comparison of the different methods of numerical taxonomy employed; list of clusters recognized by the different methods*

Original method of Pohja (1960)		Method of Sneath (1957*b*)		Method of Silvestri *et al.* (1962)	Method of Gyllenberg (1962)
Cluster	Sub-group	Test group I	Test group II		
IA	c	1	1	IA	IABC
BI	c	2	2	IBC	IABC
CI	c	2	2	IBC	IABC
IIA	d	3	3_1	IIA	IIA
IIB	d	3	3_2	IIB	IIB
IIC	d	3	3_1	IIC	IIC
IID	d	3	3_1	IID	IID
IIE	d	4	4	IIEG	IIE
IIF	d	6	*	IIF	IIF
IIG	d	4	4	IIEG	IIG
IIH	d	5	5	IIH	IIH
III	g+S†	7	*	III	*
IV	e	4	*	IV	*
VIII	f	10	*	VIII	*
IX	d	3	*	IX	*

* The method was not applied to the test group which included the strains producing this cluster.

† S, *Staph. saprophyticus*.

In selecting the clusters the following conditions were defined: the clusters should be indicated by at least two out of the four methods employed; and when indicated by the procedure of Silvestri *et al.* (1962) the homogeneous nucleus of the cluster should include at least three strains.

(c) *Design of a routine to identify halotolerant micrococci of fermented meat*

Sixty-two different tests were employed in the original work to characterize the isolates. These tests were now ranked in order of their effect in separating the clusters presented in the preceding paragraph. The rank was computed according to the method briefly described above. The following rank was obtained: production of acid from arabinose (test 17); optimum NaCl concentration for growth (test 59); optimum temperature range for growth (test 56); production of acid from

Table 4. *The development of an identification scheme for halotolerant micrococci of fermented meat origin*

Clusters	Suggested characters, from test*					Nos. of strains with characters suggested, in clusters														Totals and sub-groups§
	17	59	56	32	7	IA	IBC	IIA	IIB	IIC	IID	IIH	IIEG	IIF	III	VIII	IV	X†	Y‡	
IA	+	−	+	+	+	7														7 c
IA	+	−	+	−	+	3	1											2		6 c
IBC	+	−	+	+	−	4	6											1	1	12 c
IBC	+	−	+	−	−		2											1		3 c
—	−	−	+	+	−	2													4	6 c
—	+	−	−	−	+	1														1 c
—	−	−	+	−	−	1													3	4 c
IIA, IID, IIEG	+	+	+	+	+			6	5	8	3		1					2		25 d_1
IIA	−	+	+	+	+			1											1	2 d_1
IIB, IIEG	+	+	+	−	+				8	1			1						1	11 d_1
IIC, IID, IIH, IIEG	+	+	+	+	−			1	2	4	1	4	2					2		16 d_1
—	−	+	+	+	−			1										1	2	4 d_1
IIEG	+	+	+	−	−								13						1	14 d_2
—	+	+	−	−	+				1					2				1	1	5 d_3
—	+	+	−	+	+						3		1	2	1					7 d_3
IIF	+	+	−	−	−									6				1	1	8 d_3
—	+	+	−	+	−		1							2				1	1	5 d_3
III, VIII	−	+	−	−	+										8	1		2	8	19 Sgf
VIII	−	+	−	+	+						1					2				3 Sgf
—	−	+	+	−	+										1	1		1	8	11 Sgf
—	−	−	−	−	+										1			1	3	5 Sgf
IV	−	+	+	−	−												3		5	8 e
—	−	−	+	−	+													2	1	3
—	−	+	−	−	−													2	3	5
—	−	+	−	+	−													1		1
Totals						18	10	9	16	13	8	4	18	12	11	4	3	21	44	191

* Test 17, acid from arabinose: +, acid; , no acid. Test 59, optimum NaCl concn: −, 5%; +, 0%. Test 56, optimum temperature range for growth: +, 20–30°; −, 31–40°. Test 32, acid from sorbitol: +, acid; −, no acid. Test 7, liquefaction of gelatin: +, positive; −, negative.

† X, other types of halotolerant strains. ‡ Y, nonhalotolerant and halophilic strains.

§ Subgroups: the numbers of strains in various clusters included in these are enclosed in rules.

sorbitol (test 32); ability to liquefy gelatin (test 7); occurrence of urease activity (test 13); etc. This rank was computed on the basis of data obtained by the study of test group I, and the rank then suggested the combinations of characteristics indicated in Table 4 (left hand columns) for identification of the different clusters. In order to check the reliability of the 'identification combinations', the distribution of the whole original material among them was also tested. As can be seen from Table 4 the distribution actually observed agrees well with that predicted. Table 4 shows, moreover, that there is quite good agreement between the results of numerical taxonomy and the 'identification combinations' obtained by means of the ranking method. However, all the clusters recognized by numerical taxonomy seem not to be separable by an identification routine which employs a set of five tests. On the other hand, an increase in the number of tests in the identification scheme does not improve the possibilities for separation of the clusters because it will also increase variation, and thus effect distribution of the strains from a given cluster among several 'identification combinations'.

On the basis of the material presented in Table 4 it seems that the clusters IA, IB and IC may not easily be distinguished from each other. Similarly, the clusters IIA, IIB, IIC, IID and IIH seem not to be separable. Clusters IIE and IIG separate from these because of their characteristic failure to attack sorbitol and to cause liquefaction of gelatin. Cluster VIII might possibly be separable from cluster III, but the present material is too limited to provide decisive evidence on this point.

As a summary of facts it seems that in routine work 6 subgroups of the halotolerant micrococci of meat origin can be recognized.

(1) Subgroup c of Pohja (1960), i.e. the clusters IA, IB and IC. This has acid production from arabinose, an optimum NaCl concentration for growth of 5% and an optimum temperature range for growth of 20°–30° as distinctive identification characteristics. We refer to this subgroup as c.

(2) Subgroup d of Pohja (excluding the clusters IIE, IIG and IIF). Organisms in this subgroup have the following distinctive identification characteristics: acid from arabinose, an optimum NaCl concentration of 0% and an optimum temperature range for growth of 20°–30°. Most strains produce acid from sorbitol and attack gelatin. This subgroup will be referred to as d_1.

(3) Clusters IIE and IIG of Pohja's subgroup d. These have the following distinctive identification characteristics: acid from arabinose, an optimum NaCl concentration of 0% and an optimum temperature range 20°–30°. All strains fail to produce acid from sorbitol and to attack gelatin. This subgroup is referred to as d_2.

(4) Cluster IIF of Pohja's subgroup d. Organisms of this subgroup have the following distinctive identification characteristics: acid production from arabinose, an optimum NaCl concentration of 0% and an optimum temperature range for growth of 31°–40°. Most strains fail to produce acid from sorbitol and to attack gelatin. We use the label d_3 for this subgroup.

(5) This subgroup includes Pohja's original subgroups f and g and several strains identified as *Staph. saprophyticus* as a result of their ability to produce acetoin. The distinctive identification characteristics of these organisms are: no acid

from arabinose, an optimum NaCl concentration of 0% and an optimum temperature range of 31°–40°. Most strains fail to produce acid from sorbitol, but all are able to attack gelatin. We refer to this subgroup as Sgf.

(6) Pohja's subgroup e. This subgroup has the following distinctive identification characteristics: no acid from arabinose, an optimum NaCl concentration of 0% and an optimum temperature range for growth of 20°–30°. All strains fail to utilize sorbitol and to attack gelatin. This subgroup is called e.

4. DISCUSSION

The present work, like the earlier study of Pohja (1960), has shown that by employing methods of numerical taxonomy several distinct subgroups can be recognized in the main group of halotolerant micrococci (*Staph. lactis*) which characterizes the micrococcus flora of fermented meat products. It is particularly interesting to note that fundamentally the same clusters and subgroups are found whatever technique of numerical taxonomy is applied. This provides strong evidence to support the classification suggested.

The classification based on numerical taxonomy is, however, rather subtle, and all distinctions revealed are not demonstrable by means of routine identification schemes. The identification scheme obtained for the halotolerant meat micrococci permits an identification of 6 subgroups only, whereas 12 distinct clusters were recognized by the methods of numerical taxonomy. However, the possible identification of six subgroups might suffice well enough for practical purposes.

There are several different aspects of the relationship between the subgroups described in the preceding section. If evaluated solely on the basis of numerical taxonomy, subgroup Sgf can be distinguished from the bulk of the other subgroups (Fig. 1). The identification scheme (Table 4) again shows a difference in salt requirements for optimal growth between subgroup c and the others, from which subgroups d_3 and Sgf then separate due to their higher optimum temperature for growth. Finally, the work of Gyllenberg & Hackman (1961) on the host range of a bacteriophage lyzing halotolerant micrococci showed that all strains of the present subgroups d_1, d_2, d_3 and Sgf were susceptible, whereas resistant strains were found only in subgroups c and e.

Although confusing at the first glance, the data referred to above may suggest the 'hierarchical scheme' presented in Fig. 2.

The question which then arises is what hierarchical level is concerned if concepts of conventional bacteriological taxonomy are applied. It seems to us that the similarity illustrated in Fig. 1 between subgroup Sgf and the other subgroups is sufficient to suggest a 'species level' for the first subdivision indicated in Fig. 2. On the other hand, the susceptibility to a common phage observed for the subgroups d and subgroup Sgf, as well as several similar features occurring in subgroup d_3 and subgroup Sgf, may point to a still closer relationship. Obviously this question remains unsolved, because no distinct reference group (e.g. one including several *Staph. aureus* strains) representing a well definable 'species level' was included in the study.

In this connection a further question arises concerning the identity of the strains primarily identified as *Staph. saprophyticus*. We suggest that no true separation between them and the other strains (i.e. those not producing acetoin) of subgroup Sgf is possible. As far as these micrococci are concerned, the production of acetoin may thus have no clear cut value as a distinctive characteristic, at least not when the present method (cf. Pohja, 1960) is used for its demonstration. Since representative reference material was lacking, we have not been able to decide whether subgroup Sgf might, nevertheless, be identical with the group referred to as *Staph. saprophyticus* by Shaw *et al.* (1951).

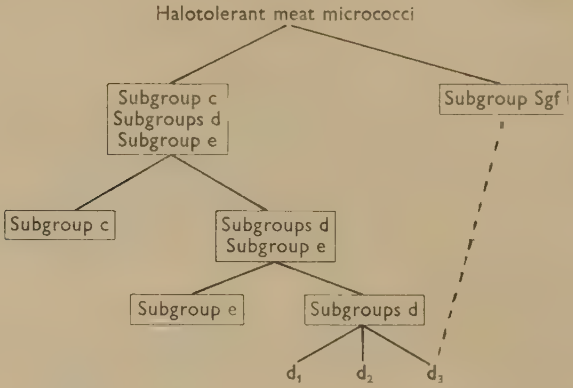


Fig. 2. A hierarchical scheme to illustrate the relationships between subgroups c, d_1, d_2, d_3, Sgf and e.

5. SUMMARY

Different methods of numerical taxonomy have been applied to the main group of 'halotolerant micrococci' (characterized by the ability to grow with both 0 and 15% NaCl in the substrate and by the lack of coagulase) which dominates the micrococcal flora of fermented meat products. All the methods applied indicated a possible subdivision of the 'halotolerant' group into several subgroups. Attempts to design an identification routine for this group showed, however, that a reliable separation of only six subgroups will be possible even with the most appropriate identification scheme. Except for one of the six subgroups, the 'halotolerant micrococci' showed characteristics which correspond to those given for *Staphylococcus lactis*. The exception was a subgroup which included strains identified as *Staph. saprophyticus*. This subgroup may possibly constitute a taxonomic group which can be distinguished from the bulk of the other subgroups.

6. REFERENCES

GYLLENBERG, H. G. and HACKMAN, C. R. (1961). A bacteriophage-lysing strain of *Staphylococcus* employed in the manufacture of dry sausage. *Appl. Microbiol.* **9**, 480.

POHJA, M. S. (1960). Micrococci in fermented meat products. Classification and description of 171 different strains. *Suom. Maataloust. Seur. Julk.* no. 96, 1.

SHAW, C., STITT, J. M. & COWAN, S. T. (1951). Staphylococci and their classification. *J. gen. Microbiol.* **5**, 1010.

SILVESTRI, L., TURRI, M., HILL, L. R. & GILARDI, E. (1962). A quantitative approach to the systematics of actinomycetes based on overall similarity. In *Microbial Classification. Symp. Soc. gen. Microbiol.* no. 12, p. 333. Cambridge: The University Press.

SNEATH, P. H. A. (1957*a*). Some thoughts on bacterial classification. *J. gen. Microbiol.* **17**, 184.

SNEATH, P. H. A. (1957*b*). The application of computers to taxonomy. *J. gen. Microbiol.* **17**, 201.

SNEATH, P. H. A. (1962). The construction of taxonomic groups. In *Microbial Classification. Symp. Soc. gen. Microbiol.* no. 12, p. 289. Cambridge: The University Press.

SOKAL, R. R. & MICHENER, C. D. (1958). A statistical method for evaluating systematic relationships. *Kans. Univ. sci. Bull.* **38**, 1409.

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Baird-Parker, A. C. (1962). *J. appl. Bact.* **25** (3), 352–361.

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THE OCCURRENCE AND ENUMERATION, ACCORDING TO A NEW CLASSIFICATION, OF MICROCOCCI AND STAPHYLOCOCCI IN BACON AND ON HUMAN AND PIG SKIN

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1. Introduction

The number of classifications which have been proposed for micrococci and staphylococci indicates the difficulty of finding a satisfactory classification (Cowan, 1962*b*). Two schemes, that outlined in the 7th Edition of Bergey's *Manual* (Breed, Murray & Smith, 1957) and that of Shaw, Stitt & Cowan (1951), were tested with 1,259 staphylococci and micrococci isolated from bacon and from human and pig surfaces. The classification in Bergey's *Manual* was unsatisfactory, as the characters of many micrococci failed to fit recognized species. All the isolates could be classified according to the diagnostic scheme of Shaw *et al.* (1951), but most belonged to only two subgroups. In developing a scheme for classifying staphylococci and micrococci from bacon and other sources we used many of the characters employed in the two previously mentioned classifications. Comparison of 56 morphological and physiological characters of the isolates indicated that the best differentiation of staphylococci from micrococci was obtained by using the ability of the former to grow and form acid from glucose anaerobically (Evans, 1948). Further division of these organisms was possible and 6 subgroups were recognized in the genus *Staphylococcus* and 7 in the genus *Micrococcus* (Baird-Parker, 1963).

In this paper we are concerned with the subgroups of staphylococci and micrococci found in vacuum packed bacon sliced from blocks cured in fresh brine, and with those occurring on human and pig surfaces. Further details of the flora and chemical changes occurring during storage of the bacon are contained in a paper by Cavett (1962).

2. EXPERIMENTAL TECHNIQUES

(a) *Isolation and maintenance of cultures*

Of the organisms isolated, 883 micrococci and staphylococci were obtained from fresh brine cured smoked bacon which was vacuum packed and stored at 20° and 30°. Two blocks of bacon were studied, one containing 5–7% and the other 8–12% of sodium chloride, based on the aqueous phase. The bacon was sliced and packed at the factory, and the packets numbered so that the position of each packet in relation to the original block was known. Four to six packets from the ends of the block were discarded to avoid possible variations in counts due to uneven distribution of salt and other curing ingredients. Packets were randomized and four packets from each block sampled immediately on delivery, which was by refrigerated van. Two packets were then sampled after storage for 2, 6, 9, 15 and 22 days at 20° or 30°. The bacon (*c.* 65 g) was macerated aseptically in distilled water, diluted with 0·1% peptone water and 0·1 ml of each dilution spread on quadruplicate plates of tryptone-yeast-glucose-salt agar (TYGSA) containing 6% (w/v) of sodium chloride (Cavett, 1962). The plates were incubated for 3 days at 30° and the number of catalase positive colonies determined on 2 plates using 3% hydrogen peroxide (Manual, 1957). One of the remaining plates was divided into 8 equal sectors and 25 catalase positive colonies picked from opposing sectors; where pickings were made from part of a sector they were picked from one edge in a clockwise direction. This procedure was followed in order to obtain a random sample of colonies.

A further 209 staphylococci and micrococci were isolated from the armpits and hands of 40 human volunteers. Hands were washed to remove transient organisms (Hurst, Stuttard & Woodroffe, 1959) and any organisms remaining on the skin were then rinsed off and cultivated on nutrient or blood agar. Transient organisms were considered unlikely to occur on armpits so no special precautions were taken to wash the skin prior to sampling.

Finally, 167 organisms were isolated from washed and ground up skin obtained from the back of the pastern joint of 30 pigs' trotters.

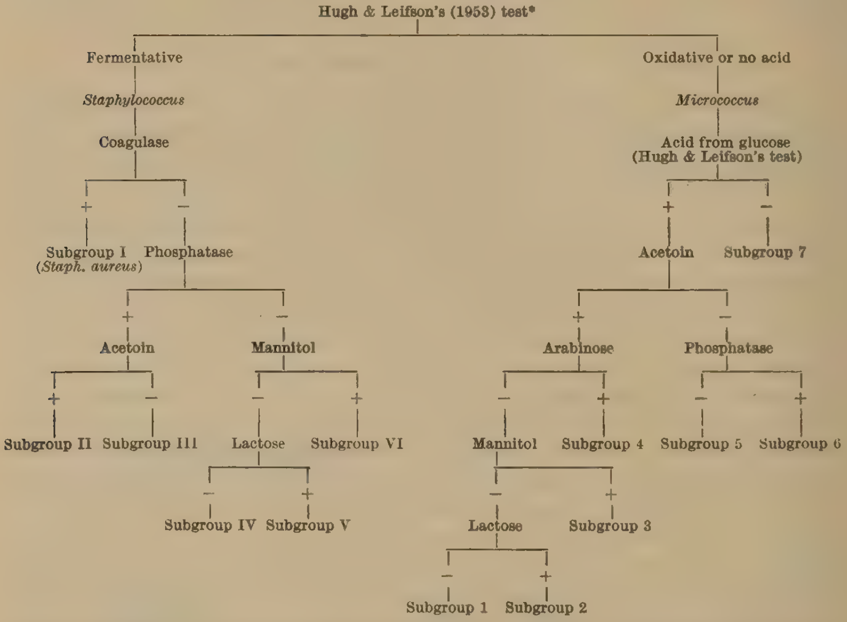
Isolates were purified on nutrient agar containing 1% of glucose. The catalase test was repeated on this medium to eliminate pediococci, which are unable to split hydrogen peroxide under these conditions and thus can be differentiated from the catalase positive micrococci and staphylococci, which produce catalase in the presence of glucose (Felton, Evans & Niven, 1953). Suspected yeasts were tested by plating cultures onto streptomycin-penicillin-malt agar. Isolates were stored on yeast-glucose agar slopes at 5°.

(b) *Classification of isolates*

Details of media and methods used are contained in another paper (Baird-Parker, 1963). A key based on this classification is outlined in Table 1.

Table 1. *A key to the separation of staphylococci and micrococci*

(From Baird-Parker, 1963)


Hugh & Leifson's (1953) test*
Fermentative
Staphylococcus
Coagulase
+
−
Subgroup I (*Staph. aureus*)
Phosphatase
+
−
Acetoin
Mannitol
+
−
−
+
Subgroup II
Subgroup III
Lactose
Subgroup VI
−
+
Subgroup IV
Subgroup V
Oxidative or no acid
Micrococcus
Acid from glucose (Hugh & Leifson's test)
+
−
Acetoin
Subgroup 7
+
−
Arabinose
Phosphatase
−
+
−
+
Mannitol
Subgroup 4
Subgroup 5
Subgroup 6
−
+
Lactose
Subgroup 3
−
+
Subgroup 1
Subgroup 2

* Modified medium used: Hucker's (1924) mineral salt base + 0·2% of agar + 0·1% of yeast extract (Difco) + 1·0% of glucose + 0·004% of bromocresol purple as indicator.

In Table 2 the possible relationships of the subgroups to the classification in Bergey's *Manual* (Breed, Murray & Smith, 1957) and to that of Shaw *et al.* (1951) are indicated.

Table 2. *The possible relationships of proposed subgroups to the named species of two recent classifications*

Bergey's *Manual* (1957)	Baird-Parker (1962)	Shaw *et al.* (1951)
	Staphylococcus	
Staph. aureus	subgroup I	*Staph. aureus*
Staph. epidermidis	subgroup II	*Staph. saprophyticus*
	subgroup III	*Staph. lactis*
	subgroup IV	*Staph. saprophyticus*
	subgroup V	
Staph. albus ?	subgroup VI	
	Micrococcus	
M. caseolyticus; *M. candidus*; *M. conglomeratus*; *M. luteus*; *M. freudenreichii*; *M. varians*	subgroup 1	*Staph. saprophyticus*
	subgroup 2	
	subgroup 3	
	subgroup 4	
	subgroup 5	*Staph. lactis*
	subgroup 6	
M. flavus	subgroup 7	*Staph. afermentans*

All the subgroups could be related to the groupings proposed by Shaw *et al.* (1951). The first 5 subgroups of staphylococci could be placed into the *Staphylococcus* spp. recognized in Bergey's *Manual*; the sixth subgroup fitted none of the currently defined species. The identity of this subgroup with *Staph. albus* will be discussed later. None of the *Micrococcus* subgroups, with the possible exception of subgroup 7, could be related to *Micrococcus* spp. in the *Manual*.

3. RESULTS

(a) *Subgroups of staphylococci and micrococci found in bacon*

The micrococcus-staphylococcus isolates from the unstored samples of the high and normal salt content vacuum packed bacon comprised 97% micrococci and 3% staphylococci. *Micrococcus* subgroup 5 was most frequently isolated (Tables 3 and 4).

Staphylococcus subgroups II, IV and VI occurred in small numbers together with members of *Micrococcus* subgroups 1, 2, 3, 4 and 7. Changes in the incidence of subgroups during storage were similar for both the high and the normal salt bacon.

Table 3. *The distribution of staphylococci and micrococci in high salt* bacon stored at 20° and 30°*

Genus	Sub-group	Percentage distribution of organisms on bacon										
		Un-stored	Stored at 20° for (days)					Stored at 30° for (days)				
			2	6	9	15	22	2	6	9	15	22
Staphylococcus	I	0	0	0	0	0	0	0	0	0	0	0
	II	0	0	0	0	3	0	56	59	77	85	77
	III	0	0	0	0	0	0	0	0	0	0	0
	IV	2	2	0	0	0	0	6	2	9	3	5
	V	0	0	2	0	0	0	0	0	2	3	10
	VI	1	2	0	0	3	0	16	0	7	0	5
Micrococcus	1	2	0	0	0	0	0	0	0	0	3	0
	2	2	0	0	0	0	0	0	0	0	0	0
	3	6	10	4	23	6	3	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0	0
	5	86	86	94	77	88	97	22	39	5	6	3
	6	0	0	0	0	0	0	0	0	0	0	0
	7	1	0	0	0	0	0	0	0	0	0	0
Nos. of isolates		88	49	49	43	36	36	50	45	43	31	39
Total count of micrococci and staphylococci (10^5/g)		0·11	1·40	70·6	88·3	77·9	71·8	179	37·4	126	136	131

* Salt concentration of 8–12% based on aqueous phase.

In bacon kept at 20° the subgroup 5 micrococci remained predominant. At 30°, however, the micrococci were replaced largely by staphylococci belonging to subgroup II. Small numbers of *Staphylococcus* subgroups III, IV, V and VI were also isolated. A single strain of *Sarcina lutea* was isolated from the high salt bacon which had been stored for 22 days at 20°.

Table 4. *The distribution of staphylococci and micrococci in normal salt bacon* stored at 20° and 30°*

Genus	Sub-group	Percentage distribution of organisms on bacon										
		Un-stored	Stored at 20° for (days)					Stored at 30° for (days)				
			2	6	9	15	22	2	6	9	15	22
Staphylo-coccus	I	0	0	0	0	0	0	0	0	0	0	0
	II	3	0	0	3	0	0	56	85	82	79	88
	III	0	0	0	0	0	4	0	3	0	0	0
	IV	0	0	3	9	0	0	7	3	6	7	0
	V	0	0	0	0	0	0	0	0	0	0	0
	VI	0	0	0	3	0	28	5	0	6	0	6
Micro-coccus	1	8	0	0	0	0	0	0	3	0	0	0
	2	3	0	0	0	0	4	0	3	0	7	6
	3	1	2	0	0	0	7	5	0	0	7	0
	4	1	0	0	0	0	4	0	0	0	0	0
	5	84	98	97	85	100	53	27	3	6	0	0
	6	0	0	0	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	0	0	0
Nos. of isolates		76	48	40	34	14	28	43	33	30	13	15
Total count of micrococci and staphylococci (10^5/g)		0·11	20·1	178	249	142	62·2	179	240	349	297	176

* Salt concentration of 5–7% based on aqueous phase.

(b) *Subgroups of staphylococci and micrococci found on human and pig skin*

Staphylococci formed the main part of the staphylococcus-micrococcus flora of human and pig skin. Although the proportions of staphylococci isolated from the two sources were similar, i.e. 84·5% for the human skin and 82·5% for the pig skin, the percentages of isolates belonging to each subgroup and the types of subgroups found were somewhat different (Table 5).

The main subgroups isolated from human skin were subgroups II, IV and VI. On pig skin, however, subgroups II and III usually predominated and organisms

Table 5. *The distribution of staphylococci and micrococci on human and pig skin*

Genus	Sub-group	Percentage occurrence on	
		Human skin	Pig skin
Staphylo-coccus	I	0·5	8
	II	36	29
	III	0	36
	IV	10	0·5
	V	4	3
	VI	34	6
Micro-coccus	1	1·5	2
	2	0·5	0·5
	3	4	3
	4	0·5	0
	5	2	3
	6	0	3
	7	7	6
Nos. of isolates		209	167

belonging to subgroups IV and VI were less common. Members of subgroup III were not isolated from human skin. Only one of the 209 isolates from human skin was coagulase positive whereas from pig skin, 13 out of 167 isolates were coagulase producers (*Staphylococcus* subgroup I).

The small numbers of *Micrococcus* isolates were distributed between most subgroups. Members of subgroup 6 were found only on pig skin. Subgroup 7 occurred on both pig and human skin and most of the tetrad forming micrococci could be placed within this subgroup. Organisms forming regular packets of cells, i.e. sarcinae, were infrequent and only two strains, both from human skin, were isolated.

A comparison of the staphylococci and micrococci from the armpits and hands indicated that similar types were present in both sites, with the exception that *Staphylococcus* subgroup VI was more frequent on hand surfaces. When comparing the floras from different subjects, however, quite large differences were found in the predominant subgroups.

4. Discussion

Staphylococci can be separated from micrococci by their ability to grow and produce acid from glucose anaerobically (Evans, 1948; Evans, Bradford & Niven, 1955; Baird-Parker, 1963). They are Gram-positive and catalase positive and form small cells of regular size; they are generally found on skin. Micrococci are usually large celled, irregular in size, form irregular clusters or tetrads, and are generally found on inanimate surfaces. Staphylococci also usually grow at 45° but not at 10°, are generally resistant to lysozyme and will initiate growth below pH 5·0 (Baird-Parker, unpublished data). Micrococci, on the other hand, will usually grow at 10° but not at 45°, are generally rapidly lyzed by lysozyme and will not grow below pH 5·0.

In placing staphylococci and micrococci into subgroups rather than species we have followed Shaw, Stitt & Cowan's (1951) procedure; this nomenclature is preferable in view of our limited knowledge as to what constitutes a bacterial species (Cowan, 1962*a*). The 13 subgroups recognized in this study form the best separation of the 1,231 isolates examined. There is evidence suggesting that certain of the subgroups, namely *Micrococcus* subgroups 5 and 7, can be further divided. Pohja (1960), and Pohja & Gyllenberg (1962) have made a detailed study of organisms corresponding to subgroup 5 and, using the Adansonian classification, concluded that this subgroup could be further subdivided. Subgroup 7 of the present work contains a miscellaneous collection of organisms forming little or no acid from glucose. However, insufficient organisms have been studied for a definite conclusion to be reached.

In the following sections aspects of the results relating to the distribution, origin and classification of staphylococci and micrococci found on bacon, human and pig skin surfaces are discussed.

Micrococci and staphylococci of bacon

The literature relating to staphylococci and micrococci in bacon is almost all concerned with the Wiltshire cure (Garrard & Lochhead, 1939; Ingram, 1955, 1960; Kitchell, 1958, 1962). Difficulties in the taxonomy of these organisms has resulted in the use of a variety of classifications for separating isolates. For example, Garrard & Lochhead arbitrarily divided their 'micrococci' into 3 main groups according to whether they were Gram-positive, Gram-variable, or Gram-negative and recognized a number of physiological types within these groups. More recently Kitchell (1958), using Shaw, Stitt & Cowan's (1951) classification, found that of 23 isolates from immature Wiltshire bacon (bacon sampled on removal from the brining tanks), 19% belonged to subgroup 2, 75% to subgroup 3 and 6% to subgroup 5. With slime from mature bacon, 98% of isolates belonged to subgroup 3 and 2% to subgroup 5. It is impossible to relate these results directly to immature fresh brined bacon as insufficient organisms have been studied from this product. However, when isolates from the unstored vacuum packed bacon were classified according to the scheme of Shaw *et al.*, 14·5% belonged to subgroup 2, 85% to subgroup 3 and 0·5% to subgroup 5. It thus appears that, although the counts of these organisms on unstored, vacuum packed, fresh brined bacon are much lower than on Wiltshire bacon (Cavett, 1962), the distribution of nonhalophilic staphylococci and micrococci in the two products is similar. Further evidence for this view is the similarity between the results obtained for vacuum packed bacon stored at 20° and 30° and those obtained by Ingram (1960) for mature Wiltshire bacon stored at 15° and 37° in cans containing either air or 95% of nitrogen plus 5% of oxygen, the latter storage condition being comparable to conditions in an unstored vacuum pack. Ingram observed that at 37°, both in air and in semi-anaerobic conditions, the normal psychrophilic flora of bacon was replaced by a mainly mesophilic one. This change was characterized by a rapid increase of the 44° and phosphatase positive counts during storage for 2 days. The phosphatase positive, but coagulase negative organisms possessed the morphology of staphylococci and undoubtedly belonged mainly to the author's *Staphylococcus* subgroup II. In vacuum packed bacon stored at 30° members of the latter subgroup have been shown to replace the mainly *Micrococcus* subgroup 5 types which occur in unstored bacon and in bacon stored at 20°.

The predominance of micrococci on freshly vacuum packed bacon is in contrast to the finding in one series of experiments of a predominance of coagulase negative staphylococci on pork blocks prior to curing (Baird-Parker, unpublished data). This change in flora is unlikely to have resulted from contamination from the brine, as freshly prepared brine was always used and the tanks frequently washed (Cavett, 1962). Counts of organisms in fresh brines are low; in used brine mainly *Micrococcus* subgroup 5 was found. The increase of micrococci over staphylococci during the curing process is a reflection of the ability of the micrococci to grow at the temperature of the cure (5°). Staphylococci are generally unable to grow even at 10° (Baird-Parker, 1963).

Micrococci and staphylococci of human and pig skin

According to Price (1938) the skin flora can be divided into 'resident' organisms which grow on skin secretions and 'transient' organisms which are unable to maintain themselves indefinitely on the skin and can be removed by scrubbing and washing. The human hand and pig skin isolates studied were obtained from washed skin and can be regarded therefore as a sample of the 'resident' flora. Pillsbury & Kligman (1954) suggested that because of the anatomical and physiological differences between skin from various parts of the body, the flora of skin could not be regarded as uniform. Therefore, two anatomically different sites, the armpits and hands, were studied in human subjects; in pigs, only skin from the back of the pastern joint was studied. The corneum of pig skin is similar to that of human skin but functional sweat glands are absent (Moritz & Henriques, 1947). The floras of pig and human skin are believed to be similar (Woodroffe, pers. comm.).

The staphylococci and micrococci of human skin have been studied by a number of workers (Pillsbury & Nichols, 1946; Evans, Smith, Johnston & Giblett, 1950; Pillsbury & Rebell, 1952; Strauss & Kligman, 1956). They have classified their isolates largely according to Bergey's *Manual*. Thus, Evans *et al.* (1950), in a study of 130 'micrococci' from normal skin, found that more than 50% of their isolates were *M. epidermidis*, 25% were *Staph. albus* and a small number were *M. candidus* and *M. flavus*. In the 7th edition of the *Manual* (Breed, Murray & Smith, 1957) the first two organisms are placed in the genus *Staphylococcus*: the organism previously called *Staph. albus* is placed with *Staph. aureus*. However, *Staph. aureus* is defined as normally coagulase positive and fermenting both glucose and mannitol under anaerobic conditions. In this present study many white, yellow or orange pigmented staphylococci were isolated which formed acid from mannitol aerobically but not anaerobically. They do not belong to any of the *Staphylococcus* species in Bergey's *Manual* but are probably identical with the organisms called *Staph. albus* by Evans *et al.* (1950) and *M. albus* (*aurantiacus*) by Pillsbury & Rebell (1952). They are members of the *Staphylococcus* subgroup VI of the present work. Further differences between this subgroup and *Staph. aureus* are the absence of phosphatase and coagulase (Baird-Parker, 1963). It is suggested, therefore, that *Staph. aureus* and the coagulase negative *Staph. albus* types present on skin have been incorrectly combined and that *Staph. albus* should be recognized as a separate species possessing those characters shown by *Staphylococcus* subgroup VI.

Staph. aureus has been isolated frequently from human skin (Gillespie, Devenish & Cowan, 1939; Williams, 1946; Hare & Ridley, 1958; Lowbury, 1961). It appears, however, to be mainly a 'transient' organism on normal skin (Pillsbury & Nichols, 1946; Ricketts, Squire & Topley, 1951). This is supported by the present results, which showed only very small numbers of *Staph. aureus* on the washed skins studied (Table 4). However, the organisms may become 'resident' under some conditions (Lowbury, 1961).

Little information has been found relating to the skin flora of the pig. Richou, Pantaleon, Billon & Cazaillet (1959) found a single strain of *Staph. aureus* among 14 'micrococci' isolated from pig skin. In this present study a similar proportion of *Staph. aureus* was isolated from washed and ground up pig skin. This organism appeared to be part of the 'resident' flora of the skin of pigs as it was isolated in large numbers from some skin samples. In the absence of details of phage typing it is impossible to say whether these organisms are of human or animal origin; physiologically they are similar to types found on humans. Smith & Crabb (1960) found the same phage types of *Staph. aureus* on the skin of pigs as on their handlers.

Staphylococcus subgroups II and III are the main coagulase negative staphylococci found on pig skin. In the bacon studied, however, mainly members of *Staphylo-*

coccus subgroup II were isolated and *Staphylococcus* subgroup III organisms were found on only two occasions. Assuming that the flora of skin from the trotter is a sample of that found on other parts of the animal, then the infrequency of subgroup III organisms in bacon could be due to several causes. First, contamination by the skin flora is avoided by processing and removal of the rind; or second, subgroup III organisms are destroyed during curing; or third, they cannot multiply in the bacon. It appears that as subgroup III organisms were not found on a small series of pork blocks examined prior to curing (Baird-Parker, unpublished data) the removal of the rind may have eliminated this organism together with the other pig skin flora. Thus chance of contamination of the bacon with *Staph. aureus* from the skin of the pig is likely to be considerably reduced by removal of the rind. The small numbers of staphylococci present on bacon, i.e. mainly subgroups II, IV and VI, probably result largely through handling and aerial contamination.

5. SUMMARY

In all, 1,259 isolates of staphylococci and micrococci were obtained from bacon and from human and pig skin; these could be classified into 13 subgroups. In fresh vacuum packed bacon, sliced from blocks cured in fresh brine, mainly *Micrococcus* subgroup 5 was found. On storage of the bacon at 20° the distribution of staphylococci and micrococci remained similar to that found on the unstored product. However, in vacuum packed bacon stored at 30° the micrococci were replaced largely by staphylococci belonging to *Staphylococcus* subgroup II.

From pig and human skin, staphylococci were isolated in larger numbers than micrococci. *Staphylococcus* subgroups II and III predominated on pig skin whereas *Staphylococcus* subgroup III was not isolated from the human skins examined and *Staphylococcus* subgroups II and VI were isolated most frequently. The identity of *Staphylococcus* subgroup VI with *Staph. albus* is discussed.

6. ACKNOWLEDGMENTS

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7. REFERENCES

BAIRD-PARKER, A. C. (1963). A classification of micrococci and staphylococci based on physiological and biochemical tests. *J. gen. Microbiol.* **29**, (in press).

BREED, R. S., MURRAY, E. G. D. & SMITH, N. R. (1957). Bergey's *Manual of Determinative Bacteriology*, 7th ed. London: Baillière, Tindall & Cox.

CAVETT, J. J. (1962). The microbiology of vacuum packed sliced bacon. *J. appl. Bact.* **25**, 282.

COWAN, S. T. (1926*a*). The microbial species—a macromyth? In *Microbial Classification. Symp. Soc. gen. Microbiol.* no. 12, p. 433. Cambridge: University Press.

COWAN, S. T. (1962*b*). An introduction to chaos, or the classification of micrococci and staphylococci. *J. appl. Bact.* **25**, 324.

EVANS, C. A., SMITH, W. M., JOHNSTON, E. A. & GIBLETT, E. R. (1950). Bacterial flora of normal human skin. *J. invest. Derm.* **15**, 305.

EVANS, J. B. (1948). Studies of staphylococci with special reference to the coagulase-positive types. *J. Bact.* **55**, 793.

EVANS, J. B., BRADFORD, W. L. & NIVEN, C. F. (Jr.) (1955). Comments concerning the taxonomy of the genera *Micrococcus* and *Staphylococcus*. *Int. Bull. bact. Nomen. Taxon.* **5**, 61.

FELTON, E. A., EVANS, J. B. & NIVEN, C. F. (Jr.) (1953). Production of catalase by pediococci. *J. Bact.* **65**, 481.

GARRARD, E. H. & LOCHHEAD, A. G. (1939). A study of bacteria contaminating sides of Wiltshire bacon with special consideration of their behaviour in concentrated salt solutions. *Canad. J. Res.* **17**, 45.

GILLESPIE, E. H., DEVENISH, E. A. & COWAN, S. T. (1939). Pathogenic staphylococci: their incidence in the nose and on the skin. *Lancet* **ii**, 870.

HARE, R. & RIDLEY, M. (1958). Further studies on the transmission of *Staph. aureus. Brit. med. J.* **i**, 69.

HUCKER, G. J. (1924). Studies on the Coccaceae. II. A study of the general characters of the micrococci. *Tech. Bull. N.Y. St. agric. exp. Sta.* no. 100.

HUGH, R. & LEIFSON, E. (1953). The taxonomic significance of fementative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J. Bact.* **66**, 24.

HURST, A., STUTTARD, L. W. & WOODROFFE, R. C. S. (1960). Disinfectants for use in bar-soaps. *J. Hyg., Camb.*, **58**, 159.

INGRAM, M. (1955). Factors controlling the occurrence of various bacteria in semi-preserved meats. *Ann. Inst. Pasteur, Lille*, **7**, 136.

INGRAM, M. (1960). Bacterial multiplication in packed Wiltshire bacon. *J. appl. Bact.* **23**, 206.

KITCHELL, A. G. (1958). The micrococci of pork and bacon and of bacon brines. *Int. Symp. Fd Microbiol.*, p. 191. London: H.M.S.O.

KITCHELL, A. G. (1962). Micrococci and coagulase negative staphylococci in cured meat and meat products. *J. appl. Bact.* **25**, 416.

LOWBURY, E. J. L. (1961). Skin disinfection. *J. clin. Path.* **14**, 85.

MANUAL (1957). *Manual of Microbiological Methods.* New York: McGraw-Hill.

MORITZ, A. B. & HENRIQUES, F. C. (1947). Studies of thermal injuries. II. The relative importance of time and surface temperature in the causation of cutaneous burns. *Amer. J. Path.* **23**, 695.

PILLSBURY, D. M. & KLIGMAN, A. M. (1954). *Modern Trends in Dermatology.* 2nd series. Ed. R. M. B. MacKenna. London: Butterworth & Co. Ltd.

PILLSBURY, D. M. & NICHOLS, M. S. (1946). Bacterial flora of normal and infected skin: an evaluation of various methods of performing skin cultures. *J. invest. Derm.* **7**, 365.

PILLSBURY, D. M. & REBELL, G. (1952). The bacterial flora of the skin. Factors influencing the growth of resident and transient organisms. *J. invest. Derm.* **18**, 173.

POHJA, M. S. (1960). Micrococci in fermented meat products. Classification and description of 171 different strains. *Suom. Maataloust. Seur. Julk.* **96**, 1.

POHJA, M. S. & GYLLENBERG, H. G. (1962). Numerical taxonomy of micrococci of fermented Meat Origin. *J. appl. Bact.* **25**, 341.

PRICE, P. B. (1938). The bacteriology of normal skin; a new quantitative test applied to a study of the bacterial flora and the disinfectant action of mechanical cleansing. *J. infect. Dis.* **63**, 301.

RICKETTS, C. R., SQUIRE, J. R. & TOPLEY, E. (1951). Human skin lipids with particular reference to the self-sterilizing power of the skin. *Clin. Sci.* **10**, 89.

RICHOU, R., PANTALEON, J., BILLON, J. & CAZAILLET, M. (1959). Recherches sur la détermination de l'origine et due pouvoir pathogène de staphylocoques isolés de produits carnés. *Rev. Immunol.* **23**, 31.

SHAW, C., STITT, J. M. & COWAN, S. T. (1951). Staphylococci and their classification. *J. gen. Microbiol.* **5**, 1010.

SMITH, H. W. & CRABB, W. E. (1960). The effect of diets containing tetracyclines and penicillin on the *Staphylococcus aureus* flora of the nose and skin of pigs and chickens and their human attendants. *J. path. Bact.* **79**, 243.

STRAUSS, J. S. & KLIGMAN, A. M. (1956). The bacteria responsible for apocrine odour. *J. invest. Derm.* **27**, 67.

WILLIAMS, R. E. O. (1946). Skin and nose carriage of bacteriophage types of *Staphylococcus aureus. J. path. Bact.* **58**, 259.

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Anderson, J. I. W. (1962). *J. appl. Bact.* **25** (3), 362–368.

(Symposium on Staphylococci and Micrococci: Paper IV)

STUDIES ON MICROCOCCI ISOLATED FROM THE NORTH SEA

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1. Introduction

The presence of Gram-positive cocci in sea water has been reported by ZoBell & Upham (1944), ZoBell (1946), Wood (1946, 1952, 1953), Brisou (1955) and Kriss (1959). Generally, these organisms have been found to represent only a small proportion of the bacterial flora of sea water and, perhaps for this reason, they have been inadequately studied. Many marine microbiologists isolating an organism that proves to be a Gram-positive coccus have been content with this information and have investigated the strain no further.

The work on micrococci which forms the basis of this paper is part of a wider investigation into the bacteriology of North Sea water, the details of which will be published elsewhere.

2 Materials and Methods

(a) *Collection of water samples*

Sea water samples were collected from a station 10 miles east south-east of Aberdeen at a depth of 10 m. by means of a J-Z water sampling device and a glass container (ZoBell, 1941), and kept under crushed ice until the plating procedure was commenced in the shore laboratory not more than 4 hr later.

(b) *Isolation of organisms*

Organisms were isolated by spreading 0·5 ml aliquots of sea water on the surface of previously dried agar plates and incubating at 20° for 14 days. A variety of complex media were used as one aspect of the investigation was to compare different isolation media. The medium which consistently gave highest counts contained: peptone (Difco), 2·5 g; yeast extract (Difco), 2·5 g; $FePO_4$, 0·1 g; agar (Difco), 15·0 g; 'aged' sea water, 750 ml; distilled water, 250 ml; and had a pH of 7·5 after sterilization at 15 lb/in^2 for 15 min.

Plate counts were carried out on the various isolation media using a series of water samples. About 700 isolated colonies were picked at random for purification and detailed study from three individual water samples collected at different seasons over a period of 2 years. Of these, 205 isolates proved to be micrococci.

(c) *Characterization of isolates*

All organisms isolated were subjected to a series of tests which was designed primarily to provide information about the physiological activities of marine bacteria and, hence, to give an indication of the microbiological potential of North Sea water.

General procedure. A basal medium (BM) was used for the growth and maintenance of all cultures. This was the same medium as that used for isolation of the organisms (see section 2b above), but the agar was omitted and sterilization varied as described below. To this medium agar and specific nutrients were added when necessary. BM, or BM plus agar, was sterilized at 15 lb/in^2 for 15 min, but when further nutrients were added sterilization was by steaming for 20–30 min on 3 consecutive days.

Colonial appearance. Colonies of cultures grown in Petri dishes on the surface of BM plus 1·5% of agar for 3–5 days at 20° were examined, using a plate microscope, and their characteristics recorded.

Gram reaction. Cultures grown in BM and on the surface of BM plus 1·5% of agar were stained by Gram's staining method using the modification of Kopeloff & Cohen (1928).

Oxidase reaction. The method used for this test was a slight adaptation of that described by Kovacs (1956). A strip of Whatman's no. 1 filter paper was impregnated with a 1% (w/v) solution of tetramethyl-*p*-phenylenediamine dihydrochloride and then laid on the surface of colonies on a BM agar plate. After a few seconds the paper was removed and, depending on the nature of the colonies, they either remained adhering to the agar surface or they were removed with the moist filter paper. In both cases a positive oxidase reaction was indicated by the colonies turning dark purple in 5–10 sec.

Catalase reaction. This test was carried out by allowing a few drops of a 10% (v/v) solution of 10 vol. H_2O_2 to fall on the surface of colonies on a BM agar plate and observing O_2 release. Uninoculated plates were used for control purposes.

Growth in a simple nitrogen medium. As all cultures isolated during this investigation were grown on media containing complex sources of nitrogen and growth factors, it was of interest to determine what proportion was able to grow in a much simpler medium. The medium designed for this purpose had the following composition: glucose, 5·0 g; sodium citrate, 1·0 g; sodium succinate, 1·0 g; sodium gluconate, 1·0 g; ammonium acetate, 1·0 g; potassium nitrate, 1·0 g; 'aged' sea water, 750 ml; distilled water, 250 ml. The pH was adjusted to 7·5, and sterilization was by steaming for 20–30 min on 3 consecutive days.

This medium was inoculated from a BM fluid culture using a straight wire, and incubated at 20° for up to 2 weeks. The appearance of turbidity was taken to indicate that the medium was suitable for the growth of the organism inoculated. If only a slight turbidity developed after prolonged incubation, a loopful of this culture was transferred to another tube of the same medium and once again incubated for up to 2 weeks. If a slight turbidity appeared once again, the organism was recorded as having the ability to grow in this medium.

Motility and cell morphology. All strains were examined under a phase contrast microscope immediately after growth was visible in a BM culture incubated at 20°.

Oxidative and fermentative attack on glucose. All strains were investigated for ability to produce acid aerobically and anaerobically from glucose, using the method but not the medium of Hugh & Leifson (1953); their medium was unable to support the growth of many marine bacteria even after the addition of the necessary sea salts. The medium used was BM plus 0·5% of glucose, 0·25% of agar (Difco) and 0·0015% of phenol red (all w/v), and had a pH of 7·5. Cultures were incubated at 20° for 14 days and examined daily for acid production.

Bromothymol blue, which was incorporated in the medium described by Hugh & Leifson (1953), was inhibitory to the growth of many of the organisms studied and phenol red is recommended as a more suitable indicator, especially for Gram-positive strains.

It is realized that the medium just described has a higher buffering capacity than the medium of Hugh & Leifson and as such may not be as sensitive an indicator of acid production. Nevertheless, it was judged to be advantageous to use one standard medium in which all isolates could grow well rather than to attempt to detect traces of acid produced by a few strains.

Utilization of mannitol, maltose, arabinose, galactose and xylose. These sugars were substituted for glucose in the medium described above. Cultures were incubated aerobically in all of these tests and examined daily for acid production. Many strains produced only a weak and transient acid reaction in some of these sugars, followed by an intense alkalinity.

Nitrate reduction. For this test 0·5% (w/v) of potassium nitrate (A.R.) was added to BM and the pH adjusted to 8·4. Cultures were incubated for 10–14 days before testing for nitrate reduction.

The production of nitrite was determined by adding to each culture tube a few drops of each of the following reagents (Manual, 1957): 8 g of sulphanilic acid in 1 litre of 5 N acetic acid; and 6 ml of dimethyl-α-naphthylamine in 1 litre of 5 N acetic acid. Uninoculated controls gave a faint background pink colour after the addition of these two reagents. This was probably due to traces of nitrite in the potassium nitrate. The production of nitrite by strains tested was easily distinguished from this faint positive reaction in the control tubes by the deep red or pink colour which developed. On a very few occasions, the addition of the reagents to the culture tube resulted in the development of no colouration whatsoever, indicating that all traces of nitrite had been removed. Since the addition of a little zinc dust to such tubes showed that nitrate was also absent, it was clear that the nitrite in the original medium had been reduced. It was thus possible to use this medium as a dual purpose test; if an intense red colour was produced, nitrate had been reduced only to nitrite; if a faint pink colour, equivalent to the colour of an uninoculated control tube, developed, nitrate had not been reduced; and, if no colour at all developed, nitrate had been reduced past nitrite.

The ability of an organism to reduce nitrate to gaseous end products was detected by the inclusion of a Durham tube in each tube of medium.

Action in litmus milk. This medium consisted of a 10% (w/v) solution of skimmed milk powder in distilled water, with litmus indicator added, and it was sterilized by steaming for 20–30 min on 3 consecutive days. Only marine bacteria capable of growth in the absence of sea water would grow in this medium and as such it was of little general value. It was retained in this scheme because, as will be discussed later, it enabled a group of micrococci to be distinguished.

Gelatin hydrolysis. The medium used for this test was adapted from Frazier (1926) and consisted of BM plus 1·5% of agar (Difco) and 0·4% of gelatin (both w/v), and had a final pH, after sterilization, of 7·5.

Cultures were spot inoculated on the surface of this medium in Petri dishes, and after incubation at room temperature for 3–5 days the plates were flooded with a solution of mercuric chloride in hydrochloric acid ($HgCl_2$, 15·0 g; conc. HCl, 20 ml; distilled water, 100 ml; Frazier, 1926). This solution precipitates unchanged gelatin and leaves a clear zone around the colony when gelatin has been hydrolyzed.

Starch hydrolysis. This medium was prepared, and cultures inoculated, in exactly the same way as has been described for gelatin plates, except that 0·5% (w/v) of soluble starch replaced the gelatin.

After incubation at room temperature for 3–5 days the plates were flooded with a 1:10 dilution of Gram's iodine solution. Starch hydrolyzing colonies were surrounded by clear zones.

Casein hydrolysis. The incorporation of casein into a medium containing sea water presented some technical difficulties. A 10% (w/v) skimmed milk solution was used as a source of casein and if this was added to a fluid sea water medium the casein was precipitated. To overcome this the preparation of the medium was as follows: a 10% skimmed milk solution was prepared in distilled water and sterilized by steaming; in addition 1·5% (w/v) of agar was added to BM and sterilized by autoclaving after dispensing in 15 ml amounts in screwcap containers. When the medium was required for use, 1·5 ml amounts of the skimmed milk and 15 ml of the BM agar melted and cooled to 42° were added aseptically to Petri dishes. When these two components were mixed, the agar quickly solidified and a smooth casein suspension was obtained.

Cultures were inoculated as described for gelatin hydrolysis, incubated at room temperature, and examined at intervals for up to 14 days. Casein hydrolysis was apparent by the clearing of the medium around the bacterial growth.

Lipolysis. The source of fat for this test was fresh dairy cream, sterilized at 15 lb in^2 for 15 min. BM agar was prepared and sterilized in 15 ml amounts as described for the casein medium. Before use, 1·5 ml of sterile cream was pipetted into 15 ml of the molten agar and thoroughly mixed before pouring.

Cultures were spot inoculated on this medium and incubated in diffuse daylight for 14 days. After incubation, the bacterial growth was scraped from the surface of the agar by means of a wire loop (this bacterial mass was used for pigment determination, see below) and the agar surface flooded with a saturated solution of $CuSO_4$ in water (Willis, 1960). This was allowed to stand for 20 min, when the excess solution was poured off. $CuSO_4$ reacts with fatty acids to form insoluble copper soaps which are bright blue. The appearance of this intense blue stain under the bacterial growth was taken to indicate lipolysis.

Pigmentation. All strains were grown for 14 days in diffuse daylight at room temperature on the cream agar described, and their pigmentation compared with colour charts (Standard, 1948, 1955). Colours were compared by scraping the bacterial growth from the agar surface, placing it on a strip of Whatman's no. 1 filter paper and examining in daylight.

Recording of results. A punch card system was found to be admirable for storing and correlating the data obtained for large numbers of isolates. Punch cards (no. P.2) obtained from the Copeland Chatterson Co. Ltd., Stroud, Glos. were used.

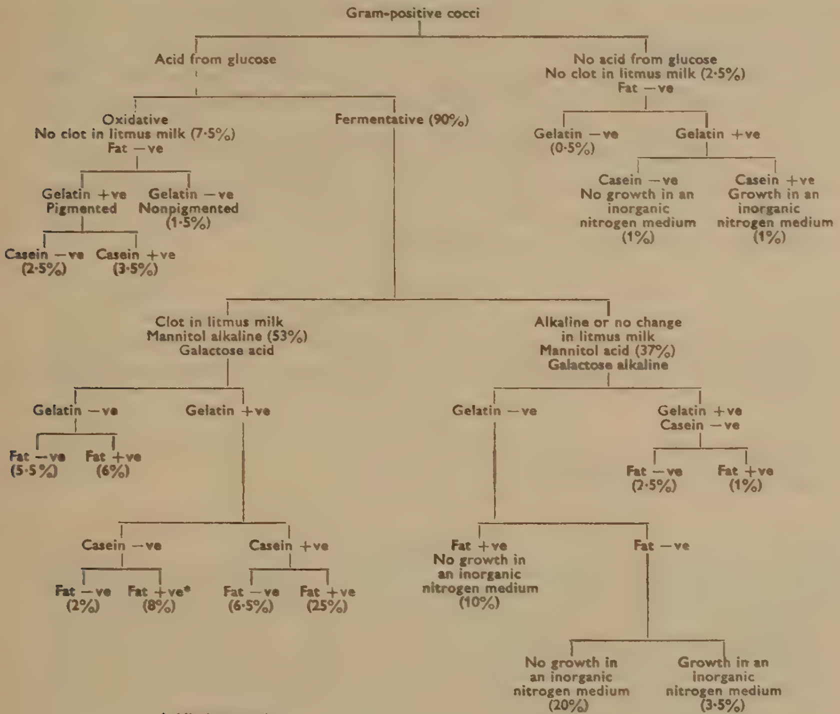
3. Results and Discussion

In this investigation 205 strains of Gram-positive, catalase positive cocci were isolated. Because of the confusion which exists at present in the classification and taxonomy of this group of bacteria they have all been placed together and designated 'micrococci'. These organisms were usually irregular in size and could range from 0·5–3·0 μ in diameter. They occurred usually in irregular clumps or clusters and occasionally in pairs. The fermentative cocci seemed to be more irregular in size than the oxidative cocci.

The 205 strains of micrococci isolated could be subdivided into 3 groups according to whether they produced acid from glucose oxidatively, or fermentatively, or not at all. It was then possible to subdivide these organisms further, into sixteen minor groups, as indicated in Fig. 1.

The fermentative cocci represented 90% of the total, and this group was divided into two subgroups. All of the strains in one of these groups produced a clot in litmus milk, a permanent acid reaction in galactose and only a transient acidity, with later alkalinity, in mannitol. Organisms in the other group failed to clot litmus milk, produced a permanent acid reaction in mannitol, and only a transient acidity in galactose. The validity of this subdivision is emphasized by the fact that the strains constituting the former group were in general much more physiologically active than those in the latter. The oxidative and the nonsaccharolytic micrococci could be grouped on the basis of gelatin hydrolysis and pigmentation but, as only a few strains are involved, these subgroups are probably of limited significance.

Certain characters proved to be of little value for systematic breakdown of the group. A fair proportion (36%) of the micrococci were pigmented, pigmentation occurring irregularly throughout the subgroups of the fermentative micrococci, except for one group which produced an acid clot in litmus milk and hydrolyzed both gelatin and fat, but not casein, and which contained only pigmented strains.


Gram-positive cocci
Acid from glucose
No acid from glucose
No clot in litmus milk (2·5%)
Fat −ve
Oxidative
No clot in litmus milk (7·5%)
Fat −ve
Fermentative (90%)
Gelatin −ve
(0·5%)
Gelatin +ve
Gelatin +ve
Pigmented
Gelatin −ve
Nonpigmented
(1·5%)
Casein −ve
No growth in an inorganic nitrogen medium
(1%)
Casein +ve
Growth in an inorganic nitrogen medium
(1%)
Casein −ve
(2·5%)
Casein +ve
(3·5%)
Clot in litmus milk
Mannitol alkaline (53%)
Galactose acid
Alkaline or no change in litmus milk
Mannitol acid (37%)
Galactose alkaline
Gelatin −ve
Gelatin +ve
Gelatin −ve
Gelatin +ve
Casein −ve
Fat −ve
(5·5%)
Fat +ve
(6%)
Fat −ve
(2·5%)
Fat +ve
(1%)
Casein −ve
Casein +ve
Fat +ve
No growth in an inorganic nitrogen medium
(10%)
Fat −ve
Fat −ve
(2%)
Fat +ve*
(8%)
Fat −ve
(6·5%)
Fat +ve
(25%)
No growth in an inorganic nitrogen medium
(20%)
Growth in an inorganic nitrogen medium
(3·5%)

* All pigmented

Fig. 1. The percentage distribution of characteristics among 205 cultures of micrococci isolated from the North Sea.

Of all the micrococci isolated, 91% reduced nitrate, 8% could grow in the simple nitrogen medium and 4% hydrolyzed starch. None produced gas from sugars and all were oxidase negative.

There is a temptation to attach considerable significance to some of the subgroups created among the micrococci isolated from the North Sea during this investigation and to give them generic or even specific status, drawing comparisons with similar treatment accorded to related organisms from other environments. This temptation has been resisted for two main reasons. First, the pattern of tests employed in this survey was designed to give information about the general physiology of the isolates and, hence, about the biological potential of the sea itself. This means that certain tests, which are primarily of a diagnostic nature and which have found application in the systematic study of individual groups from other environments, have been omitted and it is not always possible to draw comparisons with the taxa proposed by other workers. Second, in the opinion of the present author many of the problems encountered in the classification of micrococci stem from the inadequacy of the information available concerning them and this is particularly true of the marine species. Whereas it would certainly be possible to apply generic, and even in some cases specific, epithets to some of the divisions created in this work, such designations in the present state of knowledge would be valueless and, indeed, harmful, since a name once applied tends to be perpetuated in the literature long after it has become meaningless as a result of later developments.

Much more work will be necessary before an appreciation of the importance of the micrococci in the sea can be reached. Nevertheless, it would appear from this investigation that the micrococci are very active physiologically and may, on occasions, play an important role in the biology of the sea.

4. ACKNOWLEDGMENT

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5. REFERENCES

Brisou, J. (1955). *La Microbiologie de Milieu Marin*. Paris: Éditions Médicales Flammarion.

Frazier, W. C. (1926). A method for the detection of changes in gelatin due to bacteria. *J. infect. Dis.* **39**, 302.

Hugh, R. & Leifson, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J. Bact.* **66**, 24.

Kopeloff, N. & Cohen, P. (1928). Further studies on a modification of the Gram stain. *Stain Technol.* **3**, 64.

Kovacs, N. (1956). Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature, Lond.*, **178**, 703.

Kriss, A. E. (1959). *Marine Microbiology (Deep Sea)*. Moscow: Izd. Akad. Nauk SSSR.

Manual (1957). The Society of American Bacteriologists' *Manual of Microbiological Methods*. New York: McGraw-Hill.

Standard (1948). *British Standard Colours for Ready Mixed Paints*. B.S.381C: (5th impression, 1958 incorporating Amendments nos. 1, 2 and 3). London: British Standards Institution.

Standard (1955). *British Standard Colours for Building and Decorative Paints*. B.S.2660: 1955. London: British Standards Institution.

Willis, A. T. (1960). The lipolytic activity of some clostridia. *J. Path. Bact.* **80**, 379.

Wood, E. J. F. (1946). The isolation of *Sarcina ureae* (Beijerinck) Lohnis from sea water. *J. Bact.* **51**, 287.

Wood, E. J. F. (1952). The micrococci in marine environments. *J. gen. Microbiol.* **6**, 205.

Wood, E. J. F. (1953). Heterotrophic bacteria in marine environments in Eastern Australia. *Aust. J. Mar. Freshw. Res.* **4**, 160.

ZoBell, C. E. (1941). Apparatus for collecting water samples from different depths for bacteriological analysis. *J. mar. Res.* **4**, 173.

ZoBell, C. E. (1946). *Marine Microbiology*. Waltham, Mass.: Chronica Botanica Co.

ZoBell, C. E. & Upham, H. C. (1944). A list of marine bacteria including descriptions of sixty new species. *Bull. Scripps Instn Oceanogr.* **5**, 239.

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(Symposium on Staphylococci and Micrococci: Paper VI)

THE WATER RELATIONS OF STAPHYLOCOCCI AND MICROCOCCI

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1. INTRODUCTION

THE WATER RELATIONS of micro-organisms have received increasing attention in recent years and the available data, spread rather thinly over many species of bacteria, yeasts and moulds, have been reviewed in detail by Scott (1957). Interest in the water relations of staphylococci and micrococci stems from the importance of these bacteria as agents of food poisoning and food spoilage, and their ability to grow in relatively dry or concentrated environments is well known. Three traditional methods of food preservation—salting, syruping and dehydration—achieve a similar end, namely a substantial reduction in the activity of the water in the food. Thus accurate information about the water requirements of micro-organisms such as staphylococci and micrococci allows useful predictions to be made concerning the safety and stability of various foods.

It is clear that the water requirements of at least some bacteria are best defined in terms of water activity of the substrate (Scott, 1953; Christian & Scott, 1953; Wodzinski & Frazier, 1960). In this paper we review the evidence for defining the water requirements for the growth of the staphylococci and micrococci in this way. The influence of the water activity of the medium upon the composition of the cell, and the relationship of cell composition to water requirement, are also discussed.

2. THE CONCEPT OF WATER ACTIVITY

Bacteria, in common with all forms of life, require water for growth. The object in examining their water relations is to determine their minimum water requirements and their responses when water is available in excess of this minimum. Experience and nutritional considerations show that bacteria will not proliferate in pure water. It follows that any substrate, be it liquid or solid, which supports growth is, in essence, an aqueous solution. When the water content of such a solution is reduced its vapour pressure and freezing point decrease and its boiling point increases. These changes in colligative properties result from a decrease in activity of the solvent water. Clearly the same effect on the water activity may be obtained by adding solutes as by removing the solvent.

By definition, the water activity (a_w) of a solution is the ratio of its water vapour pressure to the vapour pressure of pure water at the same temperature, so that $a_w = p/p_o$. Biologists have frequently discussed solute concentrations in terms of equilibrium relative humidity (E.R.H.) or of osmotic pressure (O.P.), and the relationships between a_w and these expressions are as follows: E.R.H. (%) $= a_w \times 100$ and O.P. (atmospheres) $= (-RT \log_e a_w)/\bar{V}$, where $\bar{V}$ is the partial molal volume of water. In this discussion a_w is used to describe the status of water in the environment, since relative humidity refers more strictly to the surrounding atmosphere and osmotic pressure presupposes a membrane of specific permeability properties.

When the a_w of a solution is altered the relative concentrations of water and of solutes also change. Thus before asserting that a microbial response to a concentrated environment is an effect of a_w it is necessary to ensure that one of these accompanying changes is not, in fact, responsible. An illustration of how this has been achieved with staphylococci is given below.

3. WATER ACTIVITY AND GROWTH

The unusual tolerance of staphylococci for high concentrations of sodium chloride is well known and forms the basis of the selective media devised for these bacteria by Chapman (1946) and by Maitland & Martyn (1948). In experiments with 14 food poisoning strains of *Staphylococcus aureus*, Scott (1953) showed that this high salt tolerance was a manifestation of a very low requirement for water. He determined the lowest a_w at which growth would occur in several nutrient media, controlling

Table 1. *The minimum water activities* (a_w) *and water contents for the growth of* Staphylococcus aureus *in various media at* 30°

(after Scott, 1953)

Nutrient substrate	Solutes added to control a_w	Minimum values for growth	
		a_w	Water content (% dry wt)
Casamino Acids, yeast extract, Casitone	Salts mixture*	0·88	375
Nutrient broth	Salts mixture*	0·86	315
Nutrient broth	Sucrose	0·88	60
Nutrient broth	Sucrose 3·44 m + salts mixture*	0·86	75
Milk	None	0·86	16
Meat	None	0·88	23
Soup	None	0·86	63

* NaCl : KCl : Na_2SO_4 :: 5 : 3 : 2 moles.

water activity by adding salts or sugars or mixtures of both. Similar experiments were performed in three dried foods, reconstituted with water to give the required range of a_w. The data in Table 1 show that growth occurred at levels of a_w down to 0·88 or 0·86 in every case. In contrast, the lowest water contents at which growth was observed in the various substrates ranged from 16% to 375% of the dry weight. Growth at these a_w levels was not affected appreciably by the nature of the major solutes present, whether these were salts, sugars, or constituents of the foods themselves. Clearly the ability of these bacteria to grow in a concentrated environment is determined not by the water content or the solute concentration but by the a_w. The water requirement of such bacteria is therefore best defined in terms of the minimum a_w that will support growth.

Using a thin layer equilibration technique, Clayson & Blood (1957) obtained growth of *Staph. aureus* at a_w levels above 0·85. Earlier reports, reviewed by Scott (1953), also support his conclusion that the food poisoning staphylococci are a homogeneous group in respect of their water requirements. The few data available suggest that other species of staphylococci also exhibit a low water requirement. The minimum levels of a_w for the growth of *Staph. aureus*, *Staph. albus* and *Staph. citreus* have been recorded as 0·88, 0·89 and 0·89 respectively (Christian & Waltho, 1961).

Information about the water relations of other micrococci is fragmentary. This is a larger group than the staphylococci and it is not surprising that greater variations in water requirements have been reported. Of 17 species of bacteria examined by Burcik (1950), the only micrococcus tested, *Micrococcus roseus*, had the lowest water requirement, growing at levels of a_w down to 0·905. In a survey which included 32 strains of bacteria (Christian & Waltho, 1961), an unspecified *Micrococcus*, which grew at 0·84 a_w, had the lowest water requirement. Much more exacting were *M. roseus* and *M. lysodeikticus*, with minimum levels of a_w for growth of 0·925 and 0·94 respectively. Solar salts are a rich source of micrococci, most of which will grow in very concentrated environments. Bain, Hodgkiss & Shewan (1957), who reviewed the bacteriology of salt, quoted Petrova's report that of 10 *Micrococcus* spp. isolated from solar salt and capable of growth in salt free medium, 6 grew in a medium saturated with NaCl, 2 in 20% and 2 in 10% NaCl. The equivalent a_w levels are approximately 0·75, 0·86 and 0·93.

In spite of the variations found in the minimum levels of a_w for growth of various strains, none of the micrococci examined had a very high requirement for water. In their summary, Mossel & van Kuijk (1955) observed that micrococci (and staphylococci) frequently grow at lower a_w levels than can be tolerated by most other bacteria.

Thus far only the lowest a_w levels which will permit growth have been discussed. Also important are the quantitative effects of higher a_w levels upon the rates of growth of bacteria. Fig. 1 (from Scott, 1953) shows the relationship between a_w and rate of growth for 14 food poisoning strains of *Staph. aureus*. The a_w of the basal medium was 0·999 and lower a_w levels were obtained by adding NaCl, KCl and Na_2SO_4 in the mole ratio 5:3:2. The distinct optimum between 0·995 and 0·990 a_w is of interest. Every nonhalophilic bacterium that has been examined with sufficient care exhibits an optimum a_w, which generally lies between 0·98 and 0·995.

Since most common bacteriological media have a_w levels above 0·995 it is likely that they provide suboptimal conditions for many bacteria. Scott (1957) has suggested that at a_w levels above the optimum cells may experience difficulty in maintaining an adequate intracellular osmotic pressure. This possibility has not been examined.

Reduction of the a_w from 0·97 to 0·90 resulted in an approximately linear reduction in growth rate. The small standard errors of the mean rates, shown as vertical lines in Fig. 1, indicate that the 14 strains were homogeneous in respect of rates of growth at all a_w levels as well as in minimum water requirement. Other effects of reducing the a_w were an increase in the length of the lag phase and a reduction in the maximum cell population.

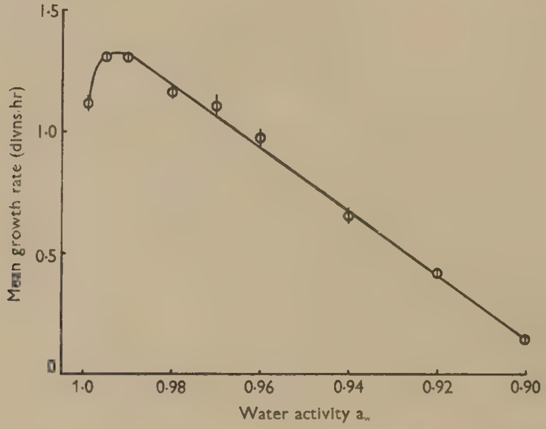

Fig. 1. The relationship between mean growth rate and water activity (a_w) for 14 strains of *Staph aureus*. Standard errors of means are shown as vertical lines. (After Scott, 1953).

To our knowledge, no quantitative data relating rates of growth to a_w have been reported for other staphylococci or micrococci.

4. FACTORS AFFECTING WATER RELATIONS

Several environmental factors have been shown to influence the water relations of staphylococci. Scott (1953) found that growth was slower at all levels of a_w under anaerobic conditions than when shaken in air. Aerobically, the minimum a_w permitting growth was 0·86; anaerobically it was above 0·90. The effects of intermediate oxygen tensions have not been studied.

All the results reviewed so far were obtained at temperatures and pH values close to the optimum for growth of the various bacteria. The relationships between these variables and salt tolerance (i.e. water requirement) of *Staph. aureus* have been reported recently (Scott, 1961). The results were only semiquantitative, being given as presence or absence of growth after incubation for 14 days. They showed that the water requirement was least at temperatures and pH values close to the normal optimum and that any substantial departure from these optima resulted in an increased requirement for water.

The data of Table 1 show no marked effects of nutrients upon the water relations of staphylococci. However, all the substrates employed were complex and unlikely to be nutritionally deficient. It has been observed that the water requirement of *Salmonella oranienburg* was much greater in a minimal medium than in rich substrates (Christian, 1955). Similar investigations with staphylococci would be of interest.

The water relations of the halophilic bacteria, a group which contains many micrococci, are complicated by their requirement for NaCl. Extremely halophilic bacteria require about 3 M NaCl and will grow in media saturated with NaCl. The moderate halophile, *M. halodenitrificans*, grows at NaCl concentrations between about 0·55 M and 4·0 M. In both cases part but not all of the NaCl requirement may be replaced by some other salts, but not by nonelectrolytes. The high minimum salt requirements result from a need for specific ions and for an adequate ionic strength, and are not primarily requirements for a low a_w level. At the other extreme, growth in the presence of 4 M or saturated NaCl certainly demonstrates a low requirement for water among the halophilic bacteria. The a_w of a saturated solution of NaCl is 0·75, and is the lowest a_w at which bacterial growth has been reported.

5. WATER ACTIVITY AND CELL COMPOSITION

The data discussed so far afford no explanation for the low water requirement of these groups of bacteria. It is probable that an almost immediate effect of reducing the a_w of the environment will be a reduction of the a_w within the cell, and hence an increase in the concentration of intracellular solutes. Measurements of the freezing points of bacteria by Christian & Ingram (1959) showed that the a_w of cells was never greater than the a_w of the growth medium. Thus the red halophilic bacteria, which grow only in media of low a_w, should possess very concentrated internal solutions. In fact, these bacteria contain sodium, potassium and chloride ions at extremely high concentrations (Christian & Waltho, 1962). The enzymes extracted from such cells are themselves halophilic (Gibbons, 1957), requiring high concentrations of salts for optimum activity. Thus the low water requirement of red halophiles is probably explained in part by the high salt tolerance of their enzymes. Similar information concerning the composition and enzyme properties of non-halophilic bacteria might assist in determining the basis of the water requirement of this group. Unfortunately little is known of the effect of a_w or concentration upon the activities of the enzymes of staphylococci, but an impression of the composition and a_w of these cells is emerging from recent work and will be reviewed briefly.

The cell of *Staph. aureus* contains a remarkably concentrated solution. Mitchell & Moyle (1956) estimated it from equilibration experiments to be osmotically equivalent to about 1 molal sucrose, or 0·98 a_w. A freezing point of −1·7° was obtained for a cell pellet containing an undefined amount of interstitial medium freezing at −0·1° (Scott, 1953). From these values it is likely that the cell a_w was slightly less than

0·98. In both cases cells were harvested from media of about 0·999 a_w. The turgor pressure was thus close to 25 atmospheres.

Partial analyses of staphylococci after growth at a_w levels from 0·993 to 0·90 were made by Christian & Waltho (paper in publication). Fig. 2 shows that the cell water content decreased from 1·66 g/g dry weight at 0·993 a_w to 0·71 g/g at 0·90 a_w. Essentially the same curve was obtained for cells grown at 0·993 a_w, washed in water and transferred to NaCl solutions of the various a_w levels prior to analysis. Fig. 2 shows the equilibrium water contents of heat killed cells at four levels of a_w. At high a_w levels these preparations took up more water than whole cells, whose expansion was limited by intact cell boundaries. At low levels of a_w the water contents were similar for each type of cell preparation, and the authors concluded that in this region of a_w cells have little direct control over their water content.

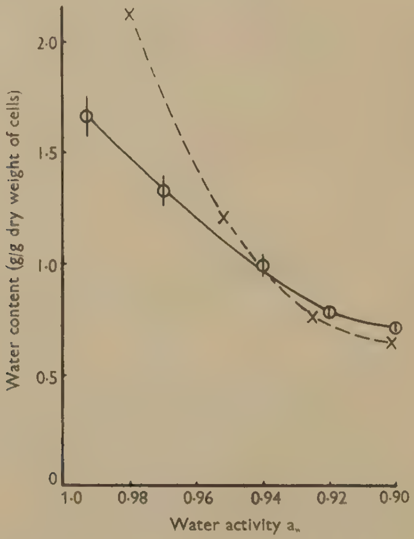

Fig. 2. The effect of water activity (a_w) on the water content of *Staph. aureus* cells. Open circles, cells grown at levels of a_w from 0·993 to 0·90. Standard errors of means are shown as vertical lines. Crosses, cells grown at 0·993 a_w, heat killed and equilibrated to the various a_w levels.

The concentrations of sodium, potassium and amino acids found in staphylococci after growth in media adjusted by the addition of NaCl to a_w levels from 0·993 to 0·90 are shown in Fig. 3. The increased sodium concentration at low a_w was partly a result of the higher sodium content of the medium. The increased concentrations of potassium and amino acids, however, were caused by the decrease in cell water content. The reciprocal changes in potassium and amino acid concentrations at low a_w are of interest. Reduction of the a_w from 0·92 to 0·90 also produced sharp decreases in the protein and inorganic phosphate contents. The authors suggest that further investigations of cell composition in this region may reveal which steps in metabolism are primarily affected by restriction of the a_w.

6. PREDICTION OF THE WATER REQUIREMENT

In a study of 32 nonhalophilic bacteria a large negative correlation was found between the potassium content of cells grown in basal medium (0·993 a_w) and the minimum a_w permitting growth (Christian & Waltho, 1961). The authors suggested that high potassium content might indicate a low intracellular a_w, which would protect the cell from dehydration or plasmolysis in a concentrated environment. The relationship between potassium content and cell a_w has been examined in the following experiments (unpublished data). Four strains, *M. lysodeikticus*, *M. roseus*, *Staph. aureus* and an unspecified *Micrococcus* sp. (designated MRI), were grown in basal brain-heart infusion broth (0·993 a_w) and analyzed for intracellular sodium, potassium, amino acids and water. The freezing points of heat killed cells were also determined. In Fig. 4 the values obtained are plotted against the minimum a_w at which growth of the respective strains occurred. The postulated relationship between potassium content and water requirement does not hold for strain MRI, which grew at a_w levels down to 0·84. However, in this organism the low potassium content appears to be compensated by an enhanced amino acid pool. It can be seen that

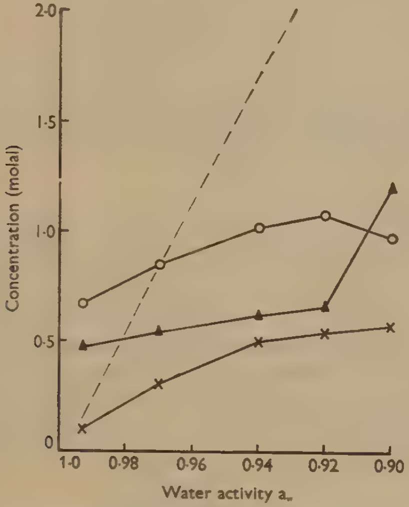

Fig. 3. The concentrations of sodium (crosses), potassium (open circles) and amino acids (full triangles) within cells of *Staph. aureus* grown in brain-heart infusion broth adjusted to levels of a_w from 0·993 to 0·90 by the addition of NaCl. The NaCl content of the medium is shown by the broken line.

as the minimum a_w decreased from 0·94 to 0·84 the sum of the intracellular sodium, potassium and amino acid concentrations increased. This suggests a concomitant decrease in intracellular a_w, which is confirmed by the freezing point depressions also shown in Fig. 4.

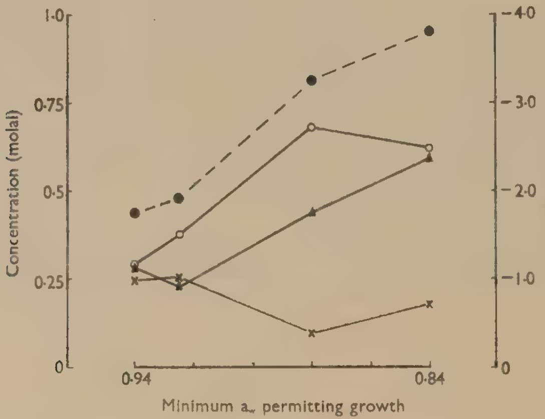

Fig. 4. The relationship between the sodium (crosses), potassium (open circles), and amino acid (full triangles) contents, and the freezing points (full circles), of four micrococci to the minimum water activity (a_w) which permitted growth. The analyses and freezing points were obtained with cells grown at 0·993 a_w.

It may be concluded that, for some micrococci at least, the minimum water requirement is related to the a_w of the cell after growth in basal medium, and that potassium content is often a reasonable guide to cellular a_w. Much more information is needed, particularly for bacteria of very high and very low water requirements, to test the validity of these conclusions. If the ability to accumulate low molecular weight solutes determines the water requirements of micrococci, the primary effects of low a_w may be upon active transport or upon the systems providing energy for transport. It is of interest that, for the four micrococci discussed here, the water requirements for respiration were similar to the water requirements for growth (unpublished data).

7. References

Bain, N., Hodgkiss, W. & Shewan, J. M. (1957). The bacteriology of salt used in fish curing. *2nd Int. Symp. Fd Microbiol., Cambridge*, p. 1. London: H.M.S.O.

Burcik, E. (1950). Über die Beziehungen zwischen Hydratur und Wachstum bei Bakterien und Hefen. *Arch. Mikrobiol.* **15**, 203.

Chapman, G. H. (1946). A single culture medium for selective isolation of plasma coagulating staphylococci and for improved testing of chromogenesis, plasma coagulation, mannitol fermentation and the Stone reaction. *J. Bact.* **51**, 409.

Christian, J. H. B. (1955). The influence of nutrition on the water relations of *Salmonella oranienburg*. *Aust. J. biol. Sci.* **8**, 75.

Christian, J. H. B. & Ingram, M. (1959). The freezing points of bacterial cells in relation to halophilism. *J. gen. Microbiol.* **20**, 27.

CHRISTIAN, J. H. B. & SCOTT, W. J. (1953). Water relations of salmonellae at 30°C. *Aust. J. biol. Sci.* **6**, 565.

CHRISTIAN, J. H. B. & WALTHO, J. A. (1961). The sodium and potassium content of non-halophilic bacteria in relation to salt tolerance. *J. gen. Microbiol.* **25**, 97.

CHRISTIAN, J. H. B. & WALTHO, J. A. (1962). Solute concentrations within cells of halophilic and non-halophilic bacteria. *Biochim. biophys. Acta* **65**, (in press.)

CLAYSON, D. H. F. & BLOOD, R. M. (1957). Food perishability: the determination of the vulnerability of food surfaces to infection. *J. Sci. Fd Agric.* **8**, 404.

GIBBONS, N. E. (1957). The effect of salt on the metabolism of halophilic bacteria. *2nd Int. Symp. Fd Microbiol., Cambridge*, p. 67. London: H.M.S.O.

MAITLAND, H. B. & MARTYN, G. (1948). A selective medium for isolating *Staphylococcus* based on the differential inhibiting effect of increased concentrations of sodium chloride. *J. Path. Bact.* **60**, 553.

MITCHELL, P. & MOYLE, J. (1956). Osmotic structure and function in bacteria. In *Bacterial Anatomy. Symp. Soc. gen. Microbiol.* no. 6, p. 150. Cambridge: University Press.

MOSSEL, D. A. A. & VAN KUIJK, H. J. L. (1955). A new and simple technique for the direct determination of the equilibrium relative humidity of foods. *Food Res.* **20**, 415.

SCOTT, W. J. (1953). Water relations of *Staphylococcus aureus* at 30°C. *Aust. J. biol. Sci.* **6**, 549.

SCOTT, W. J. (1957). Water relations of food spoilage microorganisms. *Advanc. Fd Res.* **7**, 83.

SCOTT. W. J. (1961). Available water and microbial growth. *Symp. Low Temp. Microbiol., Camden, N.J.*, p. 89. Camden, New Jersey: Campbell Soup Coy.

WODZINSKI, R. J. & FRAZIER, W. C. (1960). Moisture requirements of bacteria. 1. Influence of temperature and pH on requirements of *Pseudomonas fluorescens*. *J. Bact.* **79**, 572.

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Thatcher, F. S. & Robinson, J. (1962). *J. appl. Bact.* **25** (3), 378–388.

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FOOD POISONING: AN ANALYSIS OF STAPHYLOCOCCAL TOXINS

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1. Introduction

Investigations of staphylococcal food poisoning have been much handicapped by lack of simple tests reliably specific for detection of the toxins that cause food poisoning, or of the strains of *Staphylococcus* that produce them. The Microbiology Section of the Food and Drug Directorate of Canada has carried out studies aimed at overcoming these handicaps.

Appraisal of the literature on staphylococcal toxins, including enterotoxin, reveals substantial conflict and uncertainty. Specific areas of disagreement have been pointed out in publications from this laboratory.

A major area of conflicting evidence centred upon whether or not food poisoning reactions in cats and possibly also in humans were caused by a specific enterotoxin or by the beta-lysin or alpha-lysin. As recently as 1959, Brown, Prichard & Quilliam noted that a culture filtrate which contained alpha-hemolysin induced an *in vitro* gut stimulant activity. The implication was made that the two properties of the filtrate were related to staphylococcal food poisoning in man. Investigation of the possible role of lysins in food poisoning showed also that these factors have been poorly defined. Conflicting evidence existed as to the extent to which the alpha-lysin was also responsible for the lethal and dermonecrotic properties of staphylococcal filtrates, nor was it known to what extent the biological manifestations of the lysins might be modified by the presence of other substances, by heating, or by purification procedures.

At least a partial explanation for such lack of agreement seemed to us to involve the use of whole filtrates or of very crude preparations of toxins, both in tests for demonstrating biological expressions of toxic substances and in the preparation of antisera. Such sera would inevitably contain antibodies against various components of the filtrate. The staphylococcus filtrate is extremely complex, containing many enzymes in addition to known toxins.

Therefore, our efforts have been centred on obtaining individual preparations of biologically active components of the staphylococcus filtrate purified to the limit of our present ability.

This paper offers a brief review of salient developments from these studies.

2. ANIMAL TESTS

Dack (1956) has described the several animal species that have been used experimentally for the detection of staphylococcal enterotoxin. We have used only cats and frogs. Reactions in the frog were not sufficiently specific to retain our interest, whereas our earlier studies with cats confirmed to our satisfaction that these animals could be used, with appropriate safeguards, to detect a specific substance that was emetic to cats and which Dolman & Wilson (1940) had shown to be related to the occurrence of food poisoning in human volunteers.

A partially purified preparation received from Dr. Dack and Dr. Bergdoll of the University of Chicago, which was emetic to monkeys *per os*, gave, after removal of contaminating lysins, the same reaction in cats when administered by the intraperitoneal route as did our own partially purified haemolysin free preparations obtained from strains known to have been implicated in food poisoning. Casman (1958) has since shown that both monkeys and cats vomit in response to a common substance. Both animals have their peculiar shortcomings as test animals, but the use of monkeys offers an advantage in that a specific emetic reaction from an orally administered specimen can be obtained without heat treatment of the preparation. Alpha- and beta-lysins, administered orally, do not evoke emesis (Fulton, 1943; Dolman, Wilson & Cockcroft, 1936). A number of substances may be present in filtrates of staphylococci which can cause emesis in cats when administered by the intraperitoneal route. Factors of cost and physical problems of the housing and care of monkeys influenced our selection of cats as test animals. We have maintained a continuous supply of 50 cats over a number of years, thereby allowing some thousands of tests.

3. PROCEDURES FOR THE PURIFICATION OF TOXINS

Our early studies suggested that the better known biological activities of the staphylococcus filtrate originated from the protein components (Matheson & Thatcher, 1955; Thatcher & Matheson, 1955; Thatcher, Matheson & Simon, 1955). Hence the purification procedures used have been based upon contemporary developments in the separation of small quantities of different proteins. Progressively, we have employed precipitation at low temperatures using ethanol or salts; electrophoresis using paper (Kunkel & Slater, 1952) and later starch (Bermes & McDonald, 1956) as the supporting medium; column chromatography using calcium phosphate gel (Keilin & Hartree, 1951) or carboxymethyl cellulose (Peterson & Sober, 1956) as the adsorbent material followed by elution with phosphate buffers at various pH values or by progressive change of ionic strength at constant pH. Details of the methods applied have been described in a series of papers from this laboratory (Matheson & Thatcher, 1955; Thatcher, Matheson & Simon, 1955; Robinson, Thatcher & Gagnon, 1958; Robinson, Thatcher & Montford, 1960*a*,*b*).

The method now established as a routine is as follows.

Enterotoxin and the haemolysins are separated from most of the other components of a culture filtrate by precipitation with zinc acetate and ethyl alcohol at 0° and pH 4·0. The precipitate, which contains enterotoxin, haemolysins, proteolytic enzymes and some other proteins, is dissolved in phosphate buffer (pH 7·0), dialyzed against several changes of glass distilled water, and lypohilized. Further separation is accomplished by subjecting the lyophilized mixture to column chromatography using carboxymethyl cellulose as the supporting medium and graded levels of phosphate buffer at pH 6·0 to elute different fractions from the column. Specific fractions are collected, lyophilized and resolved still further by an electrophoretic procedure using granules of potato starch as the supporting medium and dilute buffers (0·01 M) at appropriate pH levels.

4. BIOLOGICAL TESTS

In order to allow comparative evaluations of different preparations, biological activities have been related quantitatively to the protein in the specimen, expressed as μg/ml. Protein was measured colorimetrically by the method of Lowry, Rosebrough, Farr & Randall (1951).

Biological activities were expressed as follows: haemolysis as μg of haemin freed from standard preparations of erythrocytes using the colorimetric method of Rimington (1942); proteolytic activity as the amount of tyrosine liberated from vitamin free casein using the method of Husain & McDonald (1958); dermonecrosis as the diameter of the necrotic zone developing in the skin of rabbits 5–7 days after intradermal injection. Other animal reactions were estimated after intraperitoneal injection of the test preparations. Lethality was noted by occurrence of death of mice in 5 hr; or of cats in 24 hr; emesis in cats was recorded when occurring within 20–120 min.

5. THE CARE OF CATS FOR TOXIN DETERMINATION

Special effort was made to maintain all cats in good health, and to provide careful maintenance treatment. The kindliness of treatment received during feeding and cage manipulation was often critical in providing animals that were docile and submissive to tests. Most cats were sufficiently amenable to treatment to obviate the need for technicians to wear protective gloves during injection. Upon arrival at the laboratory all animals were given a clinical examination by a veterinary pathologist. Sound specimens were placed in quarantine where they received treatment for the removal of ecto- and endoparasites and were immunized against feline enteritis. The quarantine period was usually about one month, which allowed the animals to become adapted to cage life and to a laboratory environment. At the time of test most cats were about half grown to mature. Very small kittens were not used. Individual cages were sterilized weekly, and bedding material changed daily. The cat colony, housed in an air conditioned room, was under the continuous supervision of a veterinary surgeon, our colleague, Dr. H. G. Grice.

6. SUBSTANCES CAUSING EMESIS IN CATS

No problems have been encountered with emetic reactions from media components. The components of staphylococcal filtrates that can cause emesis by the intraperitoneal route are the alpha- and beta-lysins and specific protein components which are nonhaemolytic, nondermonecrotic, nonlethal in the sense of a lethal toxin, and have no proteolytic activity. Such substances will be referred to as enterotoxins.

Early work with the use of impure preparations to immunize groups of cats demonstrated the existence of at least two and possibly three serologically distinct enterotoxins. These were obtained from diverse *Staphylococcus* strains. This laboratory has subsequently isolated lysin free preparations of three such substances. Two of them have been recovered from the same filtrate of a single strain. Some properties of these enterotoxins will be described later. The alpha- and beta-lysins, when present in sufficient concentration to evoke emesis, almost invariably cause death of the test animals. The minimum amount of beta-lysin which can cause emesis is that present in 1 ml of a solution having a titre of 1:80. We have not determined a comparable value for alpha-lysin, but boiling of a filtrate or a crude preparation containing these lysins does not leave an emetic residue of lysin, except when the lysins are initially present at very high titres, of the order greater than 1:1000, and where injections of 5 ml are administered. For experimental purposes we prefer to separate the lysins from the enterotoxins by fractionation, in order to avoid the risk of reducing the potential activity of the enterotoxins by heat treatment, unless filtrate or food preparations have been demonstrated to be free from significant amounts of lysins. Further differentiation between the effect of lysins and enterotoxins is possible. An emetic quantity of either lysin causes vomiting within about 10 min. A response from enterotoxin rarely occurs before 20 min; a period of from 30–60 min is more common.

7. THE PURIFICATION OF LYSINS

As already mentioned, the literature on the staphylococcal lysins contains many confusing statements. Purification procedures were applied to the lysins in order to facilitate the investigation of their emetic potentialities and other properties. Our interest was confined to the alpha- and beta-lysins because other lytic components of the filtrate, such as the delta-lysin, had not shown any emetic properties in cats. Our first preparations were difficult to identify as the classical alpha- and beta-lysins. We preferred to call them rabbit cell and sheep cell lysins. We believe we have been able to make some clarification here. At least two separate entities specifically lyze sheep erythrocytes and do not destroy those of the rabbit. Culture filtrates containing them demonstrate the well known 'hot cold' lytic phenomenon. Only one of the purified preparations does this. However, both sheep lysins have a positive requirement for specific divalent cations such as Mg^{++}, Mn^{++}, Co^{++} and Ni^{++} (Robinson *et al.* 1958). When these cations are present in sufficient amounts, quantitative estimation of each lysin is possible after incubation at 37°. Their infrared spectra are very similar, but an ultraviolet absorption spectrum shows a distinct difference. Their electrophoretic properties also differ, thus allowing ready separation. Both were inactivated by a carefully adsorbed preparation of beta-antitoxin kindly given us by Dr. C. E. Dolman, and each would react with an antiserum prepared from the other. Purified preparations of these lysins caused emesis in cats.

The rabbit lysin did not require cations to bring about haemolysis. The presence of chelating agents such as ethylenediaminetetra-acetic acid (EDTA) and citrate, which completely inhibited the sheep lysins, increased the lytic activity of the rabbit lysin (Robinson, Thatcher & Montford, 1960*a*). This effect may have been due to the removal of toxic ions. Addition of divalent cations would reverse the inhibitory effect of EDTA on the sheep lysin. The rabbit lysin was completely inactivated by a commercial standard alpha-antitoxin. Thus, the purified alpha-lysin lyzes rabbit cells and sheep cells only sparingly. These findings point to a source of considerable confusion in the past, since it was not customary to add the necessary cations when estimating the beta- (or sheep) lysins, and the progressive dilution system of titrating them would lead to suboptimal levels of cations. An excess of cations could reduce the activity of the alpha-lysin.

Much earlier literature needs to be assessed in the light of these findings, which may point to one source of confusion between the emetic effects of the beta-lysin and enterotoxin. By the methods in general use, the beta-lysin would not be detected in the absence of the appropriate cations.

8. THE DERMONECROTIC AND PROTEOLYTIC PROPERTIES OF SPECIFIC TOXINS

We have reported potent amounts of dermonecrotic toxins in enterotoxic cheese (Thatcher, Simon & Walters, 1956). We felt that such toxins should be investigated on the basis of purified preparations. The literature reviewed recently by Robinson, Thatcher & Montford (1960*b*) expresses two views about the dermonecrotic toxins of *Staphylococcus* spp. One is that the dermonecrotic and haemolytic effects are biological manifestations of a single substance—the alpha-lysin. Some authors, for example Leonard & Holm (1935), have stated that the inactivation of lytic properties also destroys any demonecrotic capacity. Others offer evidence to show that the use of erythrocyte stroma to adsorb alpha-lysin from a filtrate removes lytic capacity but does not interfere with the dermonecrotic potentiality (Weld & Gunther, 1931). This illustrates a basis for a second opinion that the lytic properties of the alpha-lysin are caused by a substance distinct from that which causes dermonecrosis.

Our contribution to the elucidation of this problem is the demonstration that a dermonecrotic substance which has no lytic capacity at all is present in the filtrate of many different strains of *Staphylococcus*, including many which produce alpha-lysin (Robinson, Thatcher & Montford, 1960*a*). This nonlytic dermonecrotic toxin does not cause emesis in cats. The alpha-lysin, so long as it can effect lysis, can also bring about skin ulceration until dilution prevents observation of these properties. The alpha-lysin is also the only major lethal component of the filtrate that we have ever found, though we were briefly confused about this until recognizing the second dermonecrotic toxin. This latter toxin is produced by a number of cultures of phage type 80/81. We investigated the toxin as a possible cause of the marked virulence of this phage type, but failed to demonstrate it in several cultures of 80 81 freshly isolated from cases of severe disease. We concluded that the nonlytic dermonecrotic toxin is not an important factor in disease.

Until two years ago, our best preparations of both dermonecrotic toxins, the alpha-lysin and the nonlytic one, were invariably associated with the capacity to free tyrosine from casein. We were prompted to examine dermonecrotic toxins for the presence of a proteolytic enzyme because specific snake venoms with dermonecrotic capacity had been reported to be proteolytic (Zeller, 1948). However, by a combination of chromatography and electrophoresis, each of the toxins has been resolved into two protein components, one of which is proteolytic but without the properties of dermonecrosis or haemolysis: these properties remain in the second protein. A manuscript with graphs illustrating these separations is in course of preparation. After examining the filtrates of many strains, we have found only one entity that corresponds to the alpha-lysin. Both the rabbit and sheep haemolysins are themselves subject to inactivation by the proteolytic complexes trypsin and pepsin: enterotoxins are not.

9. The Thermostability of Toxins

Several workers have shown that the alpha- and beta-lysins, when present in a culture filtrate, are inactivated to a greater degree at 60° than at 100°. Our findings agree, but the degree of sparing is small and subject to variation at different pH values.

The enterotoxins can withstand boiling when present in a culture filtrate or a food. Our experience agrees with that of Dack (1962), and his colleagues, who suggested that purification of enterotoxins renders them more vulnerable to heat. We have noticed apparent differences among the enterotoxins in their respective properties of heat resistance. We are of the opinion that controlled studies with the different enterotoxins in solution at a series of pH values is necessary for confirmation.

Dack (1962) also reported that there is some evidence that when a culture filtrate containing enterotoxin is heated the preparation may be inactive to monkeys *per os*, but is still effective by intraperitoneal injection into cats. Three possible explanations come to mind: first, different toxins may be involved; second, physiological reactivation of a partially heat damaged toxin may occur within the cat tissues; third, heat induced denaturation of the toxin protein may have occurred, thereby rendering it more vulnerable to attack by proteolytic enzymes of the intestinal tract of the monkey.

10. THE SEROLOGY AND CYTOPATHOGENICITY OF PARTIALLY PURIFIED TOXINS

Using purified preparations as antigens for the development of antitoxins in rabbits, we have demonstrated that the homologous antisera are exclusively specific, with no serological cross reactions occurring among preparations of alpha-lysin, the beta-lysins, and two enterotoxins. Our colleagues (Guerin, Jackson & Morgan, 1962) have verified this for two of our enterotoxins and the alpha-, beta- and delta-lysins.

This group of workers also examined the cytopathogenic effect of these five toxins in mammalian heart fibroblast cultures, and showed them to be distinctively different in their reactions. Dr. Morgan considers one of our enterotoxins ('Tadmore') to be highly cytopathogenic; the second (ML5) is not. The latter is the common food poisoning toxin.

Milone (1961) has subsequently reported a study of the cytopathogenic effects of the two enterotoxins, S6 and 196E, in various states of purity in an attempt to adapt tissue culture methods to a bioassay of enterotoxin. Any demonstrable cytopathogenic effects could not be directly attributable to enterotoxin. This is to be expected from the results of Dr. Morgan, who used our enterotoxin preparations of cultures ML5 and 'Tadmore', which were free from any other toxin. Dr. Casman has shown that our ML5 toxin is serologically identical with his 196E; the latter he believes to be the common food poisoning toxin (Casman, 1960, 1962). ML5 was without cytopathogenicity. Dack (1962) believes the S6 toxin to be associated with strains that cause pseudomembranous enterocolitis. Thus the cytopathogenic 'Tadmore' enterotoxin is a newly recognized addition to the staphylococcal armamentarium. It will be interesting to know if such a toxin has a role in general staphylococcal disease.

In an open discussion of the nomenclature of staphylococcal toxins held during the 1962 Annual Meeting of the American Society for Microbiology, a proposal was made to standardize the nomenclature of the three presently known toxins as A, B and C, with the type reference toxins being, respectively, those of cultures 196E (common food poisoning), 243 (enteritis), and 137 (from a culture of phage pattern 42D). We believe our ML5 to be type A, our L16 may be type B and our cytopathogenic 'Tadmore' (which is also from a culture of phage type 42D) may be type C, or perhaps a new designation, type D, will be needed. Exchange of specific antisera is desirable to determine these.

11. ENTEROTOXIN HAEMAGGLUTINATION

In our experience, anti-enterotoxic sera derived from rabbits have always been of low titre, varying from 1:4 to 1:8 (cf. Casman, 1962). Precipitin reactions by the gel diffusion method require several days for completion. In part to conserve time and sera, we have experimented with haemagglutination methods, which up to the present have been used only with our ML5 and 'Tadmore' preparations. Sheep erythrocytes sensitized with tannic acid after the method of Boyden (1951) were first used as indicator. The two sera were free from any cross reactive properties, but a nonspecific haemagglutinogen was encountered. The ensuing lack of dependability was overcome by using as indicator human cells of blood type O sensitized with formaldehyde after the method of Carter (1962). The nonspecific haemagglutinogen is first removed from the test serum with nonsensitized human O cells, after which the specific enterotoxin factor is titrated using sensitized cells. A typical response is shown by the data in Table 1. The method seems to offer promise.

Table 1. *The reaction between anti-enterotoxin and homologous enterotoxin ('Tadmore') as indicated by haemagglutination with human O erythrocytes*

Antigen	Extent of haemagglutination* with antiserum diluted					
	1:16	1:32	1:64	1:128	1:256	Undiluted
None (control)	++++	+	−	−	−	−
1/16 cat dose	++++	++++	++++	+++	−	−
1 cat dose	++++	++++	+++	−	−	−

* Haemagglutination: ++++, very marked; +++, marked; +, slight; −, absent.

12. Phage Pattern and Enterotoxin Production

Hobbs (1955) and Gentzkow (1958) have implied that the production of enterotoxin may be restricted to specific phage patterns. Our experience, involving many different phage patterns, suggests that phage pattern bears little relationship to the potentiality for forming enterotoxin. Further, cultures of the same phage pattern, but derived from different sources, have differed in their capacity to produce enterotoxin in a specific medium. Sugiyama, Bergdoll & Dack (1960) have shown similar variations between colonies of the same culture growing in a single Petri dish.

Recently Dr. Betty Hobbs gave us the opportunity to examine enterotoxin production by strains that were not typable by standard phages, but which had been isolated from cheese involved in food poisoning outbreaks. Our fractionation procedure allowed the isolation of a specific cat emetic enterotoxin from each culture. Dr. Dack informed us by letter that filtrates from the same cultures were emetic to monkeys.

13. The Formation and Demonstration of Enterotoxins in Foods

Most of the work described above has been based on the use of fractionated preparations from culture filtrates, or the filtrates themselves. However, the cat reaction and the demonstration of dermonecrosis in mice have each been used for the detection of toxins in foods. Some observations with foods baffle attempts at explanation.

Many food poisoning outbreaks appear to be the result of exposure of a contaminated food for only a few hours to temperatures favourable to the multiplication of staphylococci (Dack, 1956, 1962). We have many times isolated *Staphylococcus* strains from foods implicated in poisoning and, after demonstrating toxigenicity in culture media, have used them for surface inoculation of identical foods. We failed to detect enterotoxin more often than not, whether the tests were conducted with a suspension of the macerated food or with aqueous extracts. When toxin was recovered, an incubation period of 48 hr was usually required. Mechanical addition of a toxin to a food elicited typical reactions. We wondered if rapid toxigenesis might be a property of cultures derived immediately from infected lesions or from a carrier. Inoculation of foods with pus from staphylococcal lesions gave reactions comparable to those from cultures many years in the laboratory.

Nevertheless, from many foods implicated in food poisoning, aqueous extracts have given typical cat reactions. These foods have included cheese, dessert pies, pastry fillings, cake and frozen Chinese foods. On other occasions an aqueous extract of a food failed to provide an emetic response, but a suspension of the macerated food caused reactions indicative of high levels of toxin. Control preparations of the same kind of food and known to lack staphylococci were entirely devoid of emetic effect. Sardines canned in oil, chow-mein and cheeses have all shown this effect.

Solution or adsorption of the enterotoxin in some specific phase of the food matrix may be involved, so that aqueous extraction fails. We wish to investigate whether toxin may sometimes be present in a form which requires further activation, perhaps by specific enzymes or prosthetic groups, before being functional. Proteolytic activation of the botulinum toxin, particularly of type E, is well known.

Many foods containing large numbers of staphylococci may also produce copious amounts of lysins. For specific soft cheeses such as those of Port Salut type, the development of necrotic ulceration in mice or guinea pigs by subcutaneous injection of aqueous extracts was a useful screening procedure for detecting staphylococcal toxins therein until better methods were devised. This reaction was indicative of alpha-lysin (Thatcher *et al.* 1956). Certain heat processed dairy products containing no viable staphylococci have induced an emetic reaction in cats. The reaction may be caused by a residuum of toxin that has survived a heat treatment of the food sufficient to destroy staphylococci; or a specific 'false positive' reaction may be involved. The latter reaction, in our experience, is limited to a single emetic spasm and occurs in from 5–10 min after injection. We have not investigated the cause of this reaction.

We concluded that better procedures for the extraction of enterotoxin fractions from foods are needed, together with better knowledge of the factors conducive to the formation of toxin, including environmental and nutritive factors, the effect of commensal organisms and of their enzymes, and the possible role of lysogenic phage in the synthesis of toxin.

14. SUMMARY AND CONCLUSIONS

The cat test for food poisoning toxins is at best a compromise pending the development of procedures with greater specificity. In this regard, haemagglutination shows promise. However, with appropriate control, the cat test can detect specific enterotoxic substances in culture filtrates, in purified preparations and in foods, the latter being subject to limitations arising from problems of extracting the toxin from the foods, and from the disadvantages accruing from the inoculation of an animal with a macerate of contaminated food. The cat will also give an emetic reaction in response to alpha- and beta-lysins, a reaction usually distinguishable from that of enterotoxins in speed of response, and by death within 24 hr as a sequel to injection of an emetic amount of lysin.

At least two nondermonecrotic proteins which lyze sheep cells are comprehended by the prevailing concept of the beta-lysin. These have a positive requirement for one of a number of specific divalent cations.

The properties of alpha-lysin include the lysis of rabbit erythrocytes and to a lesser extent those of the sheep, dermonecrosis, and lethality to several test animals. It is commonly associated with a proteolytic enzyme from which it may be separated. Haemolysis is not dependent upon the presence of cations.

A nonhaemolytic dermonecrotic substance is commonly present in the filtrates of many strains of *Staphylococcus*.

At least three emetic toxins are produced by staphylococci. One of these may be the most common in food poisoning, but the full roles of these respective toxins await clarification.

These emetic toxins are distinctive proteins which are nonhaemolytic, nondermonecrotic, nonlethal in the sense of a lethal toxin, are nonproteolytic and, unlike lysins, are resistant to the proteolytic action of pepsin and trypsin. One purified enterotoxin has been shown to be strongly cytopathogenic in tissue culture. Two others lack this property. They are rather weakly antigenic but can be detected serologically by precipitin reactions or by haemagglutination.

As Dack (1962) has pointed out, supplies of specific polyvalent enterotoxin antisera would be a great aid to the detection of food poisoning toxins and of the strains of staphylococci that produce them.

More knowledge is desirable concerning the factors conducive to the elaboration of enterotoxins, their conditions of thermostability and procedures for isolating them from foods.

15. References

Bermes, E. W. & McDonald, H. J. (1956). Electro-osmosis on glass fiber filter paper. *Biochim. biophys. Acta* **20**, 416.

Boyden, S. V. (1951). The adsorption of proteins on erythrocytes treated with tannic acid and subsequent haemagglutination by antiprotein sera. *J. exp. Med.* **93**, 107.

Brown, D. A., Prichard, B. N. C. & Quilliam, J. P. (1959). Some pharmacological properties of the α toxin of *Staphylococcus pyogenes*. *Brit. J. Pharmacol.* **14**, 59.

Carter, G. R. & Rappay, D. E. (1962). Formalinized erythrocytes in the haemagglutination test for typing *Pasteurella multocida*. *Brit. vet. J.* **118**, 289.

Casman, E. P. (1958). Serological studies of staphylococcal enterotoxin. *Publ. Hlth Rep., Wash.*, **73**, 599.

Casman, E. P. (1960). Further serological studies of staphylococcal enterotoxin. *J. Bact.* **79**, 849.

Casman, E. P. (1962). A medium for the production of "F" type staphylococcal enterotoxin. *Bact. Proc.* 64.

Dack, G. M. (1956). *Food-poisoning*, 3rd ed. Chicago: University Press.

Dack, G. M. (1962). Staphylococcal enterotoxin. International Symposium on Food Protection. Iowa State University, Ames, Iowa.

Dolman, C. E. & Wilson, R. J. (1940). The kitten test for staphylococcus enterotoxin. *Canad. J. publ. Hlth* **31**, 68.

Dolman, C. E., Wilson, R. J. & Cockcroft, W. H. (1936). A new method for detecting staphylococcus enterotoxin. *Canad. J. publ. Hlth* **27**, 489.

Fulton, F. (1943). Staphylococcal enterotoxin, with special reference to the kitten test. *Brit. J. exp. Path.* **24**, 65.

Gentzkow, C. J. (1958). Staphylococcic food poisoning. *Quart. Bull. Ass. Food and Drug Off. (U.S.)* **22** (3), 112.

Guerin, M. M., Jackson, A. W. & Morgan, J. F. (1962). Tissue culture and immunological studies with purified staphylococcal toxins. *Canad. J. publ. Hlth* **53**, 38.

Hobbs, B. C. (1955). Public health problems associated with the manufacture of dried milk. I. Staphylococcal food poisoning. *J. appl. Bact.* **18**, 484.

Husain, I. & McDonald, I. J. (1958). Characteristics of an extracellular proteinase from *Micrococcus freudenreichii*. *Canad. J. Microbiol.* **4**, 237.

Keilin, D. & Hartree, E. F. (1951). Purification of horse-radish peroxidase and comparison of its properties with those of catalase and methaemoglobin. *Biochem. J.* **49**, 88.

Kunkel, H. G. & Slater, R. J. (1952). Zone electrophoresis in a starch supporting medium. *Proc. Soc. exp. Biol., N.Y.*, **80**, 42.

Leonard, G. F. & Holm, A. (1935). A method for the production of staphylococcus toxin and toxoid. *J. Immunol.* **29**, 209.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

Matheson, B. H. & Thatcher, F. S. (1955). Studies with staphylococcal toxins. I. A reappraisal of the validity of the "Kitten test as an indication of staphylococcal enterotoxin". *Canad. J. Microbiol.* **11**, 372.

Milone, N. A. (1961). On the possible use of tissue culture for the bioassay of staphylococcal enterotoxin. American publ. Hlth Ass., Laboratory Section, Detroit, Mich.

Peterson, E. A. & Sober, H. A. (1956). Chromatography of proteins. I. Cellulose ion-exchange adsorbants. *J. Amer. chem. Soc.* **78**, 751.

Rimington, C. (1942). Haemoglobinometry. *Brit. med. J.* **1**, 177.

Robinson, J., Thatcher, F. S. & Gagnon, J. (1958). Studies with staphylococcal toxins. IV. The purification and metallic requirements of specific hemolysins. *Canad. J. Microbiol.* **4**, 345.

Robinson, J., Thatcher, F. S. & Montford, J. (1960*a*). Studies with staphylococcal toxins. V. Possible identification of alpha hemolysin with a proteolytic enzyme. *Canad. J. Microbiol.* **6**, 183.

Robinson, J., Thatcher, F. S. & Montford, J. (1960*b*). Studies with staphylococcal toxins. VI. Some properties of a non-hemolytic dermonecrotic toxin produced by some strains of *Staphylococcus aureus*. *Canad. J. Microbiol.* **6**, 195.

Sugiyama, H., Bergdoll, M. S. & Dack, G. M. (1960). *In vitro* studies on staphylococcal enterotoxin production. *J. Bact.* **80**, 265.

Surgalla, M. J., Bergdoll, M. S. & Dack, G. M. (1953). Some observations on the assay of staphylococcal enterotoxin by the monkey feeding test. *J. lab. clin. Med.* **41**, 782.

Thatcher, F. S. & Matheson, B. H. (1955). Studies with staphylococcal toxins. II. The specificity of enterotoxin. *Canad. J. Microbiol.* **1**, 382.

Thatcher, F. S., Matheson, B. H. & Simon, W. R. (1955). Studies with staphylococcal toxins. III. The application of paper ionophoresis to the resolutions of components of tonic concentrates. *Canad. J. Microbiol.* **1**, 401.

Thatcher, F. S., Simon, W. & Walters, C. (1956). Extraneous matter and bacteria of public health significance in cheese. *Canad. J. publ. Hlth* **47**, 234.

Weld, J. T. P. & Gunther, A. (1931). Differentiation between certain toxic properties of filtrates of hemolytic *Staphylococcus aureus*. *J. exp. Med.* **54**, 315.

Zeller, E. A. (1948). Enzymes of snake venoms and their biological significance. *Advanc. Enzymol.* **8**, 459.

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Parker, M. T. (1962). *J. appl. Bact.* **25** (3), 389–402.

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PHAGE-TYPING AND THE EPIDEMIOLOGY OF *STAPHYLOCOCCUS AUREUS* INFECTION

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1. Introduction

Staphylococcus phage-typing is one of the ways in which medical bacteriologists have tried to subdivide the species of staphylococcus that is important to them as a human pathogen. *Staphylococcus aureus* can be found somewhere on the body surfaces of half the population, and is regularly present in the air of occupied places and on objects in contact with people. Little progress could therefore be made in studying the spread of staphylococcal infection until some method of distinguishing between the many different strains of *Staph. aureus* had been found.

2. The Development of Staphylococcus Phage-typing

The classification of staphylococci by determining their susceptibility to lysis by phage only became possible when a source of phages with a sufficiently narrow range of host specificity had been found.

Phages with a wide host range, which will lyze most coagulase positive staphylococci and often a number of coagulase negative strains as well, are easily isolated from natural sources, such as sewage, but are of little use for typing. In 1942, however, Fisk showed that most strains of *Staph. aureus* are lysogenic; that is to say, they carry phages to which they are themselves immune, but which will lyze some other members of the same species. Fisk developed a primitive method of typing by cross culture, in which a spot inoculum of one staphylococcus strain was superimposed upon a lawn of a second strain, and the appearance of plaques of lysis indicated that one of the pair carried a phage virulent for the other. The current phage-typing method is, however, a direct descendant of the scheme proposed by Wilson & Atkinson (1945). These workers chose a series of typing phages, made high titre preparations of them by propagation on staphylococci for which they were virulent, and separated them from the bacteria by filtration. In order to keep the host range of a phage constant it was always propagated on one strain of staphylococcus, referred to as its propagating strain. The strength of each phage was standardized with reference to its routine test dilution (RTD)—the highest dilution of a filtrate which gave a defined amount of lysis of its own propagating strain. In the test, drops of the phage filtrates which had been diluted to the RTD were applied to a plate freshly spread with an even inoculum of a staphylococcus, and evidence of lysis was sought after incubation. Some of the phages were obtained by cross culture from lysogenic strains of *Staph. aureus*. Others were obtained by applying strong filtrates of these phages to strains of staphylococci which were not lyzed at RTD; when plaques were seen, propagation was attempted on the new host staphylococcus with the object of adapting the phage to it. A number of true adaptations were obtained, but later serological studies indicated that some of the phages obtained in this way were unrelated to the original phages and were probably other phages carried by a strain on which propagation had been attempted. Although there is doubt about the origin of some of these supposed adaptations, several of them have proved to be useful typing phages.

Wilson & Atkinson's work was done in the years immediately following the recognition by Cowan (1939) that most human strains of *Staph. aureus* fell into one of three main serological groups, and the development by Craigie and his colleagues (Craigie & Yen, 1938; Felix, 1943) of a phage-typing system for *Salmonella typhi*. Both of these discoveries had their influence on the development of staphylococcus phage-typing. The strains which Wilson & Atkinson endeavoured to type included not only a representative collection from the main staphylococcal diseases of man, but also groups of strains of known serological type (Atkinson, pers. comm.), and this had its effect on the phages selected for the first typing system.

Craigie's Vi-phage II (Craigie & Yen, 1938) has the remarkable property of lyzing strains similar to the one on which it was last grown, and the phages used for typing *Salm. typhi* are a series of adaptations of the one phage. The host range of most of these adapted phages is very narrow, and the typing scheme is therefore a relatively simple 'diagonal' one; with the exception of members of Vi-phage type A, which are lyzed by all the typing phages, the rest of the types are characterized by lysis by a single adapted phage, with few cross reactions. Wilson & Atkinson (1945) were seeking a similar 'diagonal' typing scheme, containing a finite number of clear cut types. They failed, as have all subsequent workers, to find a single phage which could be adapted in the same way as the Vi-phage II, but with an arbitrarily selected group of 18 phages, not more than three of which were adaptations of any one original phage, they were able to type 82% of strains of *Staph. aureus*.

A strain was considered to be typable if it gave confluent lysis with one or more of the phages at RTD. Three-quarters of the typable strains could be accommodated in 21 types, 13 of which were defined by lysis by a single phage, and the others by relatively simple patterns of lysis by 2, 3 or 4 phages; the remainder of the typable strains gave more complicated patterns of lysis.

Subsequent experience has amply confirmed the practical value of Wilson & Atkinson's method, but our ideas about the nature of the subdivisions it makes have undergone substantial modification. Williams & Rippon (1952), in the Staphylococcus Reference Laboratory of the Public Health Laboratory Service, used Wilson & Atkinson's 18 phages together with six others. In order to type three-quarters of their strains they had to accept 50 or more plaques at RTD as evidence of strong lysis. Their conventions for recording the strength of reactions are still in use, and are illustrated in Plate 1. When they compared groups of strains from one source there was often a variation in the degree of lysis produced by individual phages: the reaction of a phage might be strong with one strain and moderate or even weak with another, and the strict application of a type designation based only upon strong reactions would have resulted in related strains being allotted to different types. It was clear, therefore, that the weak reactions would have to be taken into account, and that a limited number of clear cut types could not be defined. Strains could, however, be characterized by the pattern of their lysis by the typing phages, and these patterns were a good guide to the relations between strains. Indeed, for the purpose for which typing was most often used—to study the spread of staphylococci in small communities for short periods of time—the fact that the subdivisions did not have any long term validity was not important, and the ability to recognize very many different patterns was an advantage.

With the present set of typing phages, about 70% of all strains of *Staph. aureus* isolated from human sources are typable, that is to say, give a strong reaction with one phage or more at RTD. The proportion of typable strains can be increased to

Table 1. *An illustration of the method of recording and reporting phage patterns using an example taken from an outbreak of food poisoning*

Strain no. and source			Degree of lysis† of staphylococci at the routine test dilution by phages‡														Reported result*
			52	52A	79	80	3C	55	71	6	7	42E	47	53	75	77	
1	Patient A	Vomit									++		++	±	++	++	7/47/75/77/+
2		Faeces									+		++	+	+	++	47/77/+
3	Patient B	Vomit								±	++		+	±	+	+	7/+
4		Faeces									++		++	+	+	++	7/47/77/+
5	Patient C	Faeces								±	++		++	+	++	++	7/47/75/77/+
6	Food	Ham								±	++		+	+	++	++	7/75/77/+
7		Tinned peas									++	++	±		++		7/42E/75/+
8		Potato salad		++	++	±											52A/79/+
9	Cook A	Nose	±	++	++	±											52A/79/+
10	Cook B	Boil									++		++	+	+	+	7/47/+
11		Nose								±	++		++	+	+	++	7/47/77/+
12	Cook C	Nose					++	++	++								3C/55/71

* Strains nos. 1, 2, 3, 4, 5, 6, 10 and 11 form a group of indistinguishable strains, and are different from no. 7; nos. 8 and 9 are also indistinguishable.

† Degree of lysis: ++, >50 plaques; +, 20–49 plaques; ±, 1–19 plaques; no entry, no lysis. See also Plate 1.

‡ Phages 29, 3A, 3B, 54, 42D, 81 and 187 did not lyze any of the 12 strains.

over 90% if the untypable strains are also tested with the same phages at 1,000 times the strength of the RTD (RTD×1,000). Although this is a very satisfactory result, RTD×1,000 typing has its problems. The reactions are somewhat more variable than those obtained at RTD and require more skill in reading. Strong phage filtrates sometimes contain small amounts of contaminating phage derived from the propagating strain. They also frequently have an inhibitory effect on the growth of some staphylococci, and this may give rise to areas of clearing which are difficult to distinguish from confluent lysis, or confluent lysis with secondary overgrowth.

The results of phage-typing are reported as a pattern, which consists of a list of the phages by which a strain is lyzed, followed by a plus sign if weak reactions with other phages are also present (Table 1). Since the weak reactions may be important in deciding the relations between strains, the final interpretation of the results must be made in the laboratory. Reactions at RTD×1,000 are seldom reported if a strain is typable at RTD.

3. THE PHAGE GROUPS OF *STAPHYLOCOCCUS AUREUS*

Although phage-typing revealed great heterogeneity in *Staph. aureus*, there were from the beginning indications of a small number of broad subdivisions within the species. This is best illustrated with reference to the typing phages in current use (Table 2). With 22 typing phages many hundreds of different patterns of susceptibility are possible, but certain combinations occur much more frequently than others. Thus, lysis by phage 52 is much more likely to be accompanied by lysis by phage 29, 52A, 79 or 80, or a combination of these, than is lysis by other phages. Patterns

Table 2. *The lytic groups of staphylococcus typing phages which are included in the internationally agreed set of basic typing phages*

Lytic group	Phages in group								
I	29	52	52A	79	80				
II	3A	3B	3C	55	71				
III	6	7	42E	47	53	54	75	77	83A
IV	42D								
Not allotted			81	187					

Table 3. *The phage groups of* Staphylococcus aureus

Phage group of staphylococcus	Strains lyzed by one or more of the phages									
I	29	52	52A	79	80	81*				
II	3A	3B	3C	55	71					
III	6	7	42E	47	53	54	75	77	83A	81*
IV	42D									
Miscellaneous	(a) Strains lyzed by phages belonging to two or more lytic groups. (b) Strains lyzed by phage 187.									

* Strains lyzed only by phage 81 are placed in phage group I.

including 3C often also contain 3A, 3B, 55 or 71, but rarely members of the 29, 52 etc. group. Thus, it was possible to subdivide the typing phages into a small number of lytic groups, members of which appear together in typing patterns, and to divide staphylococci into corresponding phage groups (Williams & Rippon, 1952). Most human strains fall into one or other of the phage groups I, II, or III (Table 3). Strains lyzed only by phage 42D are placed in phage group IV. Two phages, 187 and 81, cannot be allotted to any of the lytic groups. Phage 81 lyzes many strains which otherwise have patterns in group I, but it also forms part of some group III patterns. Phage 187, on the other hand, lyzes strains which are sensitive to this phage only.

In addition to the strains falling into the four phage groups, a small percentage have complex patterns including phages in two or more groups; they are referred to as miscellaneous strains. Lysis by phage 81 in addition to phages of lytic groups I or III does not, however, exclude a strain from the corresponding phage group. Strains lyzed by phage 187 are almost certainly distinct from members of the four main phage groups, but are probably not common enough to warrant the establishment of a fifth group.

There is little doubt that the phage groups are reflections of more fundamental subdivisions within *Staph. aureus*. Phage groups I, II and III correspond approximately to Cowan's serological groups I, II and III (Hobbs, 1948) and members of these groups have antigenically distinct coagulases (Barber & Wildy, 1958). The production of enterotoxin is restricted in the main to phage groups III and IV (Allison, 1949; Williams, Rippon & Dowsett, 1953). There is also some evidence that members of the phage groups differ in the ease with which they become resistant to antibiotics, and in their ability to give rise to particular lesions or to initiate large outbreaks of staphylococcal infection. It is probable, however, that these differences are more closely associated with certain specific patterns of susceptibility to the phages than with the phage groups as a whole. Thus, in phage group I, the 52, 52A, 80, 81 complex of strains appears to differ from the rest of the group in its ability to give rise to outbreaks of severe sepsis in hospitals. In phage group II, strains reacting only with phage 71 appear to be specifically associated with vesicular skin lesions, such as staphylococcal impetigo and pemphigus of the newborn (Parker, Tomlinson & Williams, 1955; Parker & Williams, 1961).

4. THE STANDARDIZATION OF PHAGE-TYPING TECHNIQUES

It is clear that the recognition of a large number of patterns with 22 phages is only possible if the technique of phage-typing is carefully controlled, and if the range of variation inherent in the method is fully understood. Now that phage-typing is used in numerous laboratories throughout the world it is important that the technique should be standardized not only within one laboratory, but internationally.

The precautions taken fall into two groups: those concerned with maintaining the consistency of phage stocks, which are the responsibility of the various national staphylococcus phage-typing laboratories, and those concerned with the standardization of the typing test in the local laboratories that receive propagated phage from the national laboratories. The International Subcommittee for Staphylococcus Phage-Typing have agreed upon a standard basic set of phages, and all laboratories are encouraged to use it, even though they may wish to add other phages which appear to them to have a local importance. The International Laboratory issues to each national laboratory, once every three years, a fresh basic set of freeze dried phage preparations, together with the corresponding propagating strains. Each time the phages are propagated they must be tested, by an agreed procedure, to ensure that no significant change in their lytic spectrum has taken place. National laboratories supply propagated phage, which should have a strength of at least RTD×10,000, to local phage-typing laboratories.

In the local laboratories the strength of the phage preparations must be determined accurately at least twice a week, or before each session if typing is done infrequently. Many media are suitable for phage-typing, but each batch must be tested before use to ensure that all the phages give the correct amount of lysis on it. The results of typing should be read and recorded in a standard manner.

All national laboratories, and other typing laboratories, are invited to take part in the International Trial of Staphylococcus Phage-Typing once every two years. Full details of the internationally agreed techniques for propagation, typing and recording of results are given in a recent paper by Blair & Williams (1961).

5. The Range of Variation in the Typing Pattern of Epidemiologically Related Strains

We have alluded to the fact that small differences in the patterns of strains thought to be epidemiologically related are quite common. How big a difference must there be before we conclude that two strains are unrelated?

Williams & Rippon (1952) studied this question exhaustively for typing at RTD, and most of our ideas are derived from their work. The degree of variation among groups of strains thought to be 'the same' increased with the remoteness of the cultures from their presumed common source. Duplicate tests on one culture showed less variation than duplicate cultures from one source, e.g. single colonies picked from the primary culture plate of a nasal swab, and these in their turn showed less variation than successive cultures from one source, or groups of cultures from different sources in an acute epidemic. In prolonged epidemics in hospitals, when a strain is transmitted during many months through a series of patients or carriers, successive cultures may show even greater variations from the original patterns. However, of sets of cultures believed to be epidemiologically related, and all tested on one day, few differ by the complete loss of more than one strong reaction. This is the basis for the 'two strong differences' rule (Williams & Rippon, 1952). The way in which the rule is applied may be illustrated from Table 1, in which it will be seen that all the phage group III strains are indistinguishable except the one from the tinned peas, which differs from the rest by two strong reactions. The same rule would be applied if typing was spread over several days, but the results would be interpreted more cautiously, and in cases of doubt some of the earlier strains would be retyped along with the later ones. The stability of reactions at RTD×1,000 has been less well investigated, but the 'two strong differences' rule may probably be applied, as long as inhibition is not confused with true lysis.

This rule forms a good rough guide to the interpretation of typing results, but the experienced worker develops individual standards which also take into account the fact that certain patterns are more stable than others. This may be apparent from a comparison of the cultures themselves: two patterns linked by a series of intermediate forms are more likely to be related than two differing by the same amount but each in itself consistent. Patterns including certain combinations of phages are usually very stable (e.g. 52A/79) and certain others, particularly those in which there are a large number of reactions, are notably unstable.

Small differences between the patterns of lysis of epidemiologically related cultures occur frequently even when the greatest care is taken to standardize the phage-typing technique, and it seems that they are inherent in the method. They may be due to variation in the initial ratio of phage particles to actively multiplying staphylococci, or to differences in the physiological state of the staphylococcal culture at the time of typing. Poorly growing cultures have a reduced susceptibility to lysis by phage, and this is probably the explanation of many of the claims to have changed the phage patterns of staphylococci by rendering them resistant to antibiotics. The size of the plaques is also much influenced by the thickness of the bacterial lawn, and the inability to detect a particular reaction is sometimes due to the fact that the plaques are too small to be seen even with a hand lens. The susceptibility of many strains to lysis may be increased by heating cultures to 56° for 2 min (Ma & Mandle, 1961). In one example of this phenomenon (Asheshov & Jevons, data to be published) a group of epidemiologically related strains were untypable by the routine technique but became sensitive to phage 187 after heating; it was found that the resistance of the strains was due to interference exerted by a prophage which they carried, and that this could be overcome by heating. A similar though less impressive increase in susceptibility to lysis occurs in some strains which have been stored for a long time in the laboratory.

It is possible that more gross changes in pattern sometimes result from loss or gain of a lysogenic phage. Staphylococci are very common on the human body surface, and two or more strains can often be isolated from the same site, so that the exchange of phage material between two distinct but related organisms could occasionally take place. Strains lyzed by combinations of phages 52, 52A, 80 and 81 form a group within which large variations of pattern are seen. They have caused many outbreaks of sepsis in hospitals in the last few years and the common association in epidemics of strains lyzed by phages 80 and 81 with others having the pattern 52/52A/80/81 suggested that they might have arisen from a common source (Asheshov & Rippon, 1959). Many members of the 52, 52A, 80, 81 complex of strains carry phages which, when transferred to other related strains, change their phage pattern. Some of these phages cause a narrowing of the phage pattern (e.g. 80/81→80) but others cause a gain of up to two strong reactions (e.g. 80/81→52/52A/80/81). The mechanism of these changes is complicated (Rountree, 1959; Asheshov & Rippon, 1959; Rountree & Asheshov, 1961), but the practical conclusion is that strains lyzed by any combination of these four phages may have arisen from a common source.

It is unlikely that changes in phage pattern due to lysogenization occur very often in nature, but when a strain which has undergone such a change is endemic in a hospital it is possible for substrains to be transmitted in parallel through successive generations of patients. Thus, though two strains with patterns 80/81 and 52/52A/80/81 may well have arisen from a common ancestor some months before, their phage patterns in particular patients or groups of patients may subsequently be relatively constant, and our interpretation of the difference between them will depend on whether we suspect a recent or a remote connection between them. Experience suggests that phage patterns containing 29 or 79 in combination with other group I phages are quite distinct from the 52, 52A, 80, 81 strains, and it therefore appears that the limits within which lysogenization can change the phage pattern are narrow.

It would be wrong to give the impression that differences between the typing patterns of related strains are often sufficiently great to cause real doubt in the interpretation of results. Williams (1957) has summarized his own experience with series of strains collected over relatively short periods of time. His general conclusion is that in acute epidemics a clear decision that strains are 'alike' or 'different' can be made in all but 5–6% of instances. In series spread over longer periods the proportion of doubtful cases is likely to be larger.

6. THE ROUTINE SET OF TYPING PHAGES

In the last 10 years several of Williams & Rippon's 24 phages have been removed from the routine typing set after it had been shown that they did little to define common patterns of lysis, and a few new phages have been introduced. Since 1953, the International Subcommittee on Phage-Typing of Staphylococci has taken responsibility for deciding which set of phages is best suited for routine typing. The present routine set is that shown in Table 2.

Many of the naturally occurring staphylococcal phages have host ranges not unlike those of the typing phages, and the introduction of a new one into the routine typing set is only justified if: first, it makes a significant reduction in the percentage of untypable strains; second, it makes typable at RTD a group of strains previously only recognizable by complex patterns at RTD×1,000; or third, it divides a set of strains previously showing the same pattern into groups which are epidemiologically distinct. Preference should be given to phages which lyze strains from lesions, rather than carrier strains. It is inconvenient, however, to use more than 25 phages, which is the maximum number that can easily be applied on one plate. The difficulty is to keep the system within reasonable bounds, without undue loss of flexibility. This is best done by ensuring that one laboratory in each country—preferably the National Laboratory—uses, in addition to the routine typing set, a pool of other phages which may be of use in particular circumstances. It need only be applied to strains which are untypable with the routine set of phages, and should contain: those phages previously rejected from the routine set because they react with too small a percentage of strains; and any phages found useful for their action on untypable strains, particularly in foreign countries.

One situation in which a new phage may be useful is in investigating a disease which has not previously been studied. This is well illustrated by the example of phage 71, which was isolated by Mr. Hood in Birmingham in 1951, but found to lyze so few strains that it was placed in the pool. It was subsequently found, however, that most strains of *Staph. aureus* from staphylococcal impetigo, and a proportion from pemphigus of the newborn, were lyzed only by this phage at RTD. Phage 71 was therefore introduced into the routine typing set by the International Sub-committee in 1958 (Report, 1959).

The introduction of a new phage may also become imperative when an increasing proportion of the strains giving rise to lesions in a particular area are untypable, or give only weak and inconstant reactions with the routine phages at RTD × 1,000. This situation has arisen several times in the last ten years. Thus, in 1954 Rountree and her colleagues (Isbister, Durie, Rountree & Freeman, 1954; Rountree & Freeman, 1955) reported the appearance in Australian hospitals of an untypable strain of unusual virulence. They subsequently obtained by adaptation a phage (phage 80) which lyzed these strains, and within a few years similar strains were identified in most countries of the world. Bynoe, Elder & Comtois (1956) in Canada obtained phage 81 in similar circumstances. There is too little evidence to decide whether these '80/81' strains were carried from one country to another, or whether they arose independently in a number of places, but their recognition in many instances followed the appearance of epidemics of unusually severe infections. Members of the 52, 52A, 80, 81 complex of strains are now the commonest single cause of hospital infection in many countries, and phages 80 and 81 are included in the routine typing set.

A strain which gives rise to a severe local epidemic of sepsis may not, however, have much importance elsewhere in the world. In 1949, for example, Colbeck reported the appearance in the Winnipeg area of a very severe epidemic of breast abscess and neonatal sepsis very like those later described by Rountree in Australia. The Canadian strains were lyzed only by a phage which was subsequently given the number 57, but no later epidemic due to this strain has been reported. There is thus no reason to include phage 57 in the routine typing set, but it should be kept in reserve for testing sets of untypable strains from epidemics of sepsis.

Phage 83A is an example of a phage which has been 'on probation'. It serves to identify an otherwise untypable strain which has assumed great importance as a cause of surgical sepsis in Great Britain since 1957 (Williams & Jevons, 1961). We have also identified it among quite small series of strains received from each of 15 other countries. There is no doubt that it must now be used for routine typing, and the International Sub-Committee added it to the routine typing set in August 1962. It belongs to lytic group III.

The value of a phage for the subdivision of strains already typable at RTD is much more difficult to determine, and must be made on epidemiological rather than laboratory evidence. We have already mentioned the frequency with which strains with patterns in 52, 52A, 80, 81 are seen in hospitals. Phages which split this group further are easy to obtain, but the splinters appear to have no greater stability than those which are made by the existing phages. In our opinion, therefore, no attempt should be made to create further subdivisions on so unstable a basis.

7. Typing Animal Staphylococci

Staphylococcus phage-typing was developed by medical bacteriologists; the typing phages were chosen to lyze human staphylococci, and the phage groups were constructed to contain them. It is interesting to see what happened when attempts were made to type strains from other animals.

MacDonald (1946), using Wilson & Atkinson's phages, found that over half of the *Staph. aureus* strains from milk were lyzed only by phage 42D; such strains, it will be recalled, have since been placed in phage group IV. Smith (1948*a*) confirmed MacDonald's findings, but showed that these strains did not, in fact, belong to one 'type'. Bovine staphylococci include strains which react strongly with 42D alone, and others which react weakly, together with a number which have wide and inconstant patterns of lysis by phages of groups I and III, with or without 42D, and others that are completely untypable. Smith (1948*b*) also showed that bovine staphylococci carried a number of phages which could be used to subdivide strains lyzed by phage 42D, and to type others which were untypable with Wilson & Atkinson's set.

It is interesting to note that human staphylococci are very rarely lyzed by phage 42D alone, and indeed are seldom lyzed by it at all. For example, we find that only 0·7% of human nasal strains, and about 0·3% of strains from septic lesions, belong to phage group IV. The only occasion on which such strains are at all commonly found in human pathological material is when they are isolated from victims of staphylococcal food poisoning, and in many of these outbreaks the organism is of animal origin. Phage group IV strains accounted for 7·4% of the staphylococcal food poisoning outbreaks in England and Wales in 1959–61 in which the causative organism was identified. It appears, therefore, that phage 42D is possibly an 'escape' from the animal world. Not sufficient is known of its origin to be sure that it was once lysogenic on a bovine staphylococcus, but this is very likely.

Staph. aureus strains from sheep are in the main untypable with the human typing phages, but may be subdivided with phages isolated from ovine staphylococci (Smith, 1948*b*).

The internationally agreed set of typing phages are therefore unsuitable for typing staphylococci from several animal species, including cattle, sheep and possibly also monkeys (Smith, 1948*b*; Rountree, Freeman & Johnston, 1956), and it may be necessary to develop several distinct typing systems for different groups of animal staphylococci. At least four different sets of phages for typing bovine staphylococci have been described at various times (Smith, 1948*b*; Seto & Wilson, 1958; Coles & Eisenstark, 1959; Davidson, 1961). It is most desirable that unified systems for typing staphylococci from cattle and sheep should be agreed upon, and that the phages used for typing staphylococci from various animal species should be characterized by their lytic spectra on a common set of test staphylococci. It should then be possible to relate the various phage-typing systems, and to carry out studies of the spread of staphylococci between different animal species. Although there appears to be a fairly sharp demarcation between the staphylococci of man and of cattle, there is evidence that the dog may, under certain circumstances, be a source of staphylococci important in human disease (Pagano *et al.* 1960; Live & Nichols, 1961).

8. DISCUSSION

A typing scheme is a method of bacterial classification at the subspecific level. Most of those who take the trouble to work out such schemes have a practical object in view, and will choose for classification those criteria most useful for their particular purpose. In some bacterial groups, fine subdivisions may be made conveniently on the basis of biochemical characters, of antigenic structure, or of the ability to produce antibacterial substances, and in others methods using phage are preferred. A typing system which is useful in practice is, however, not necessarily one which will satisfy the bacterial taxonomist, who is naturally suspicious of any grouping based on a single character.

The medical bacteriologist types an organism in order to distinguish between alternative sources of infection. The questions asked of him by field epidemiologists usually take the form "Did organisms A and B arise from the same source?" or "Did A infect B?", and it is wise for him to remember that this is the one question he can never answer with certainty. The fact that two organisms belong to one type—whatever the typing method—never proves that they are epidemiologically related, though it may support an opinion, arrived at from other evidence, that they are. The strength of the support it gives will depend on our knowledge of the relative frequency with which organisms of the type are to be found in the population under investigation. On the other hand, if two organisms belong to different types, and the typing method is a good one, this is positive evidence that the one cannot have arisen from the other. Thus the crude question "The same or different?" must be rephrased more cautiously as "Distinguishable or indistinguishable?"

The end product of typing is often referred to as the 'type', but uncritical use of the word is a common cause of confusion. If a bacterial species is subdivided on one criterion, it cannot be assumed that members of the resultant 'types' will be identical in all other respects. Indeed, the fact that two typing methods split the same bacterial species into groupings which cut across one another should cause no surprise. When the subdivisions are qualitatively distinct, for example, with each characterized by one of a series of chemically related but distinct antigens, such as the pneumococcal capsular polysaccharides, it is justifiable to refer to them as types, if the term is qualified by a descriptive adjective. Thus, we may speak of serotypes, biochemical types and, in some circumstances, of phage types, but never of 'types'.

With *Staph. aureus*, however, we should be very careful about the way in which we use the term 'phage type'. We have chosen to use a method which results in very fine subdivisions of *Staph. aureus* because we are dealing with epidemiological situations in which we have to distinguish between numerous different strains. Many of our pattern reactions, though useful in studying the spread of infection, have little validity outside the outbreak under investigation.

Having said this, however, we must recognize that susceptibility to the typing phages is related, though often in a rather shadowy way, to a number of other characters of staphylococci that are of great interest to us. The phage groups I, II and III probably represent fundamental divisions among the human strains of *Staph. aureus*, and susceptibility to phages may prove a useful method of characterizing the staphylococci of certain other animal species. There are also numerous indications that particular phage patterns within groups I, II and III characterize strains with the ability to cause certain sorts of lesion, or to spread in a particular way (Williams, 1959; Williams & Jevons, 1961).

It has seldom been possible to detect *in vitro* a toxin or other agent which might be responsible for the properties of a particular staphylococcus, though it appears that strains which give large intra-epidermal vesicles are characterized by the ability to produce large amounts of hyaluronidase (Faber & Rosendal, 1960). There are, however, several cultural characters, such as the ability to cause opacity in egg yolk (Gillespie & Alder, 1952) or in horse serum (Tomlinson & Parker, 1956), to produce certain antibiotic substances (Parker, Tomlinson & Williams, 1955) or to resist the action of mercuric chloride (Moore, 1960) which appear to be 'markers' for staphylococci with certain phage patterns which have a characteristic pathogenicity or epidemic virulence. When we know more about the 'real' differences between these sorts of staphylococci we may perhaps be justified in referring to them as types.

9. SUMMARY

Phage-typing is a useful method of studying the spread of *Staph. aureus* in human populations, although it does not result in the definition of a limited number of clear cut phage types. A comparison of the patterns of susceptibility of staphylococci to the typing phages is a valuable method of identifying those strains which are epidemiologically related. Phage-typing also gives some indication that strains of *Staph. aureus* differ in their ability to cause particular lesions, and particular sorts of epidemics. The internationally agreed set of typing phages was chosen for the characterization of staphylococci causing disease in man, and it is probable that different phages would be more suitable for the typing of strains from cattle, sheep, and other animals.

10. REFERENCES

ALLISON, V. D. (1949). In discussion on food poisoning. *Proc. R. Soc. Med.* **42**, 216.

ASHESHOV, E. H. & RIPPON, J. E. (1959). Changes in the typing pattern of phage type 80 staphylococci. *J. gen. Microbiol.* **20**, 634.

BARBER, M. & WILDY, P. (1958). A study of the antigenic specificity of staphylococcal coagulase in relation to bacteriophage group. *J. gen. Microbiol.* **18**, 92.

BLAIR, J. E. & WILLIAMS, R. E. O. (1961). Phage typing of staphylococci. *Bull. World Hlth Org.* **24**, 771.

BYNOE, E. T., ELDER, R. H. & COMTOIS, R. D. (1956). Phage typing and antibiotic-resistance of staphylococci isolated in a general hospital. *Canad. J. Microbiol.* **2**, 346.

COLBECK, J. C. (1949). An extensive outbreak of staphylococcal infections in maternity units. Use of bacteriophage typing in investigation and control. *Canad. med. Ass. J.* **61**, 557.

COLES, E. H. & EISENSTARK, A. (1959). Staphylococcic phages. III. Typing of *Staphylococcus aureus* cultures isolated from the bovine udder. *Amer. J. vet. Res.* **20**, 838.

COWAN, S. T. (1939). Classification of staphylococci by slide agglutination. *J. Path. Bact.* **48**, 169.

CRAIGIE, J. & YEN, C. H. (1938). The demonstration of types of *B. typhosus* by means of preparations of type II Vi phage. *Canad. publ. Hlth J.* **29**, 448, 484.

DAVIDSON, I. (1961). A set of bacteriophages for typing bovine staphylococci. *Res. vet. Sci.* **2**, 396.

Faber, V. & Rosendal, K. (1960). Correlation between phage-type and hyaluronidase-production of *Staphylococcus aureus*. *Acta path. microbiol. scand.* **48**, 367.

Felix, A. (1943). Experiences with typing of typhoid bacilli by means of Vi bacteriophage. *Brit. med. J.* **i**, 435.

Fisk, R. T. (1942). Studies on staphylococci. II. Identification of *Staphylococcus aureus* strains by means of bacteriophage. *J. infect. Dis.* **71**, 161.

Gillespie, W. A. & Alder, V. G. (1952). Production of opacity in egg-yolk media by coagulase-positive staphylococci. *J. Path. Bact.* **64**, 187.

Hobbs, B. C. (1948). A study of the serological type differentiation of *Staphylococcus pyogenes*. *J. Hyg., Camb.*, **46**, 222.

Isbister, C., Durie, E. B., Rountree, P. M. & Freeman, B. M. (1954). A further study of staphylococcal infection of the new-born. *Med. J. Aust.* **ii**, 897.

Live, I. & Nichols, A. C. (1961). The animal hospital as a source of antibiotic-resistant staphylococci. *J. infect. Dis.* **108**, 195.

Ma, P. & Mandle, R. J. (1961). Phage typing of non-typable strains of *Staph. aureus*. *Bact. Proc.* p. 111.

MacDonald, A. (1946). *Staphylococcus aureus* in cows' milk: the results of phage typing. *Mon. Bull. Ministr. Hlth Lab. Serv.* **5**, 230.

Moore, B. (1960). A new screen test and selective medium for the rapid detection of epidemic strains of *Staphylococcus aureus*. *Lancet* **ii**, 453.

Pagano, J. S., Farrer, S. M., Plotkin, S. A., Brachman, P. S., Fekety, F. R. & Pidcoe, V. (1960). Isolation from animals of human strains of staphylococci during an epidemic in a veterinary school. *Science* **131**, 927.

Parker, M. T., Tomlinson, A. J. H. & Williams, R. E. O. (1955). *Impetigo contagiosa*. The association of certain types of *Staphylococcus aureus* and of *Streptococcus pyogenes* with superficial skin infections. *J. Hyg., Camb.*, **53**, 458.

Parker, M. T. & Williams, R. E. O. (1961). Further observations on the bacteriology of impetigo and pemphigus neonatorum. *Acta paediat., Stockh.*, **50**, 101.

Report (1959). International Committee on Phage-Typing of Staphylococci. *Int. Bull. bact. Nomen. Taxon.* **9**, 115.

Rountree, P. M. (1959). Changes in the phage-typing patterns of staphylococci following lysogenization. *J. gen. Microbiol.* **20**, 620.

Rountree, P. M. & Asheshov, E. H. (1961). Further observations on changes in the phage-typing pattern of phage type 80/81 staphylococci. *J. gen. Microbiol.* **26**, 111.

Rountree, P. M. & Freeman, B. M. (1955). Infections caused by a particular phage type of *Staphylococcus aureus*. *Med. J. Aust.* **ii**, 157.

Rountree, P. M., Freeman, B. M. & Johnston, K. G. (1956). Nasal carriage of *Staphylococcus aureus* by various domestic and laboratory animals. *J. Path. Bact.* **72**, 319.

Seto, J. T. & Wilson, J. B. (1958). Bacteriophage typing of micrococci of bovine origin. *Amer. J. vet. Res.* **19**, 241.

Tomlinson, A. J. H. & Parker, M. T. (1956). Production of opacity on serum agar plates by some strains of *Staphylococcus aureus*. *Nature, Lond.*, **177**, 895.

Smith, H. W. (1948*a*). Investigations on the typing of staphylococci by means of bacteriophage. I. The origin and nature of lysogenic strains. *J. Hyg., Camb.*, **46**, 74.

Smith, H. W. (1948*b*). The typing of staphylococci of animal origin by the bacteriophage method. *J. comp. Path.* **58**, 179.

Williams, R. E. O. (1957). Die Beständigkeit der durch Lysisbilder bestimmten Staphylokokkentypen. *Zbl. Bakt.* (Abt. 1) **168**, 528.

Williams, R. E. O. (1959). Epidemic staphylococci. *Lancet* **i**, 190.

Williams, R. E. O. & Jevons, M. P. (1961). Lysotypen von *Staphylococcus aureus* verschiedener Herkunft. *Zbl. Bakt.* (Abt. 1) **181**, 349.

Williams, R. E. O. & Rippon, J. E. (1952). Bacteriophage typing of *Staphylococcus aureus*. *J. Hyg., Camb.*, **50**, 320.

Williams, R. E. O., Rippon, J. E. & Dowsett, L. M. (1953). Bacteriophage typing of strains of *Staphylococcus aureus* from various sources. *Lancet* **i**, 510.

Wilson, G. S. & Atkinson, J. D. (1945). Typing of staphylococci by the bacteriophage method. *Lancet* **i**, 647.

EXPLANATION OF PLATE

PLATE 1, (a)–(d). Examples of phage lysis at the routine test dilution (magnification ×3). Conventions used for recording the strength of lysis are as follows:—

- (a) Strong reaction, confluent lysis (Wilson & Atkinson, 1945).
- (b) Strong reaction, ++ (> 50 plaques) (Williams & Rippon, 1952; and subsequent workers).
- (c) Moderate reaction, + (20–49 plaques).
- (d) Weak reaction, ± (1–19 plaques).

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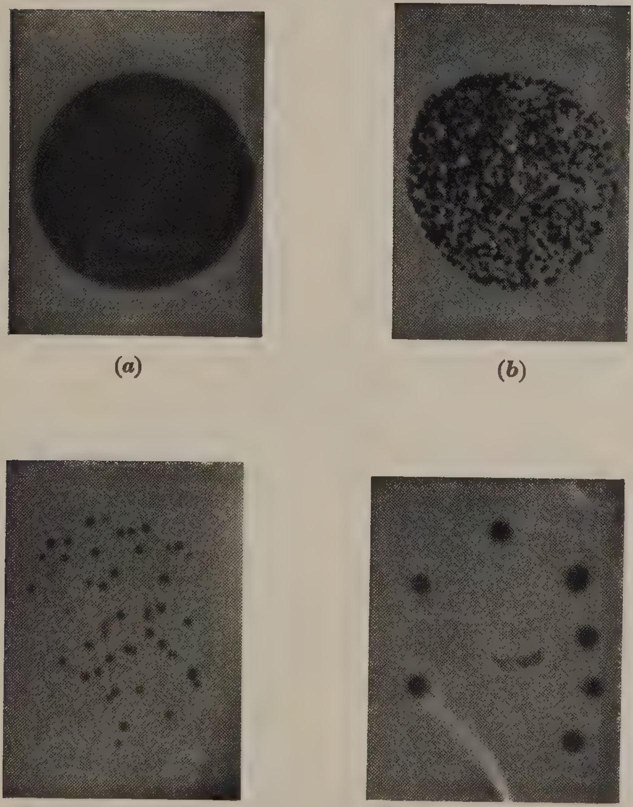

(*a*) (*b*)

(*c*) (*d*)

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STAPHYLOCOCCI AND MICROCOCCI ASSOCIATED WITH DAIRYING

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1. INTRODUCTION

FOR THE PURPOSE of this work the classification of Abd-el-Malek & Gibson (1948) has been the most convenient to use. According to this, organisms originating from the animal body and those from dairy equipment can be distinguished as staphylococci and micrococci, respectively, by their heat resistance, production of NH_3 from arginine, formation of acetoin, and fermentation of mannitol. The staphylococci are further divided into four subgroups, one of which contains coagulase positive strains, and corresponds to *Staphylococcus aureus* of Shaw, Stitt & Cowan (1951) and will be referred to here as *Staph. aureus*. The others consist of coagulase negative strains.

At the present time, *Staph. aureus* is the most frequent cause of udder infection and is excreted with the milk of infected cows. Coagulase negative strains also commonly infect the udder and both types are frequently present on the teats and udder of the cow, whereas micrococci are common on dairy equipment. These organisms are therefore always present in commercial raw milk. Unless the milk is adequately heat treated, *Staph. aureus* can survive, and under favourable conditions multiply, in both liquid milk and dairy products. Some strains of these organisms are known to produce enterotoxin so that any dairy product severely contaminated with coagulase positive staphylococci is potentially dangerous to man, and indeed outbreaks of food poisoning due to dairy products do sometimes occur (Report, 1958*a*; Smith, 1961; Allen & Stovall, 1960; Hausler, Byers, Scarborough & Hendricks, 1960). This paper deals with staphylococcal infection of the cow, where contamination of the milk usually originates, and with the concentration and survival of *Staph. aureus* in Cheddar cheese. It also refers to coagulase negative staphylococci, their production of penicillinase, and their possible relation to the development of typical Cheddar flavour.

2. STAPHYLOCOCCI OF THE UDDER

Although the most common form of staphylococcal mastitis infection is mild or chronic, infection may result in severe illness or even death of the cow. The use of penicillin has reduced the incidence of the more infectious streptococcal mastitis and has left the field open to the staphylococci. These organisms are less easily eliminated from the udder by antibiotic treatment, and owing to a selection of strains resistant to antibiotics they are becoming still more difficult to remove. A recent examination at the Institute of 604 cows from different herds showed that at least 54% of them were infected with staphylococci, 40% had *Staph. aureus* and 17% had coagulase negative staphylococci. Some samples had both kinds. Most of the udders infected with *Staph. aureus* eventually develop mastitis, whereas those infected with coagulase negative strains seldom do so. In addition to intramammary infection, staphylococci occur frequently on the clean udder and teats, eroded teat apices being particularly susceptible to colonization by diphtheroids and coagulase negative staphylococci. *Staph. aureus* also occurs at this site and may colonize the eroded teat canal and persist for several months without necessarily causing an intramammary infection. Whether this is due to individual resistance of the animal

Table 1. *The extent of contamination of milk with* Staphylococcus aureus *arising from infected, eroded teat sphincters**

Cow no.	Nos. of *Staph. aureus*/ml of milk† or /swab from		
	1st oz of milk taken with a sterile cannula	2nd oz of milk drawn by hand	Swab of teat sphincter
1	0	200	>5,000
2	0	480	
3	0	1,000	
4	0	4,400	
5	0	520	
6	0	1,400	

* The cows' udders and teats were disinfected by swabbing with sodium hypochlorite (800 p/m) or cetyltrimethylammonium bromide (1% w/v).

† All samples of milk were negative to the Whiteside test.

or to lack of invasiveness of the strain, or to some other factor, is not known. When the erosion is severe or there is an open sore at the orifice, a sample of milk may contain several thousand staphylococci/ml even though the udder itself is not infected. Table 1 shows the contamination of milk with *Staph. aureus* growing in eroded teat sphincters but not present in the udder. Staphylococci are not recovered when a sterile cannula is used to withdraw the milk, but they occur in large numbers when the milk is withdrawn normally. Thus large numbers of staphylococci may be secreted into the milk by a cow free from an intramammary infection.

McEwen & Cooper (1947) listed the most common microorganisms causing udder infection according to the frequency of infection of the udder and the proportion of infections of each type giving rise to mastitis, their observations being in agreement with ours in that the coagulase positive staphylococci were very much more pathogenic than the coagulase negative strains. The latter seldom cause mastitis and have thus received much less attention than the coagulase positive ones.

One hundred coagulase negative staphylococci isolated from infected quarters of cows and from eroded teat canals of noninfected animals were found by us to fall into group 1B or 1C of Abd-el-Malek & Gibson (1948), and are thus closely related to the coagulase positive group 1A.

(a) *Isolation of staphylococci from teats and udders*

As these organisms are usually present in pure culture or in association with diphtheroids in infected udders, their isolation is simply a question of collecting milk samples aseptically and plating on 7% calf or sheep blood agar. Colonies of *Staph. aureus* are usually easily distinguished from other staphylococci by the type of haemolysis formed, although doubtful colonies should be confirmed by the coagulase test. With a few exceptions, described by Garvie & Cheeseman (1958) and by Garvie, Higgs & Neave (1961), all staphylococcus colonies producing dark zones characteristic of staphylococcus β-lysin are in fact *Staph. aureus*. For the isolation of staphylococci from a mixed flora, which will occur when bulked milk or teat swabs are examined, the 8% salt-blood agar of Simpkin, Higgs & Neave (unpublished data) consisting of nutrient agar plus 8% of NaCl and 8% of calf blood was found to be preferable to tellurite or phenolphthalein phosphate media.

On this salt blood medium all organisms except staphylococci are almost completely suppressed. Strains producing β-lysin are as clearly differentiated as on blood agar, by large cloudy zones, so that no further confirmation of these as *Staph. aureus* is necessary. Fortunately most bovine strains of *Staph. aureus* produce β-lysin and this medium is a useful aid in their rapid identification. However, a number of *Staph. aureus* strains produce only α-haemolysin, and this lytic effect may be partially or completely suppressed on this medium, whereas some produce neither α- nor β-lysin. On plates containing a mixed flora such staphylococcal colonies must be confirmed by a coagulase test. Where it is necessary to look for small numbers of staphylococci from contaminated sources, such as the skin of teats, enrichment may be necessary. Incubation of the sample or swab in litmus milk for 24–48 hr at 37° followed by streaking on salt-blood agar was found to be more efficient than enrichment in milk or broth containing 6–14% of NaCl.

The numbers of *Staph. aureus* in the milk samples from infected udders may show great fluctuations from none to 10^8/ml, even within 24 hr, but are usually about 10^4/ml.

(b) *Differentiation between strains of* Staph. aureus

As with the human types, *Staph. aureus* of bovine origin may be classified into a number of serological groups or into a large number of phage types. They may also be classified according to the number of toxins or lysins formed.

There is a general relationship between the source of a strain and the predominating toxin formed *in vitro*. For instance, whereas poultry strains produce little or no haemolysin (Smith, 1954) human strains produce predominantly α-lysin and most bovine strains mainly β-lysin. Phage type and source are also related: ovine strains usually differ from those commonly recovered from humans. Within any one herd of cows there may be a complete relationship or no relationship, between phage type, types of haemolysin produced and colour of growth on blood agar.

Table 2. *Some characteristics of strains* of* Staph. aureus *isolated from cases of mastitis*

Strain	Penicillinase production†	Haemolytic titre of broth cultures of		No. of flocculation lines in nutrient agar	Degree of infectivity‡	Degree of pathogenicity§	Phage pattern
		Laboratory culture	Fresh isolate**				
d	−	139	175	5	++	+++	70/W6/78
m	+	143	82	8	++	++	6/7/47/54/75/77/52B
t	+	2	1	6	++	+	42D/42F/S20/710
c	−	18	19	5	+	+	29/52/52A/52B/70
f	−	109	89	8	+	+	42D/42F/S20/710/W7

* All strains were phosphatase positive. † Penicillinase: produced, +; not produced, −. ‡ Infectivity: ++ significantly more infectious than +. § Pathogenicity: +++ significantly more pathogenic than ++; ++ significantly more pathogenic than +.

** Laboratory cultures were used to produce a natural infection in cows and the fresh isolates were obtained from this source.

In some instances a general relationship has been observed between the nature of the infection in man and the phage type (Parker, Tomlinson & Williams, 1955). This has not been sufficiently studied in the cow although a few strains, selected on the basis of phage type, have been shown to differ in their ability to infect the udder, skin, and eroded teat apices, even though little difference was found in their pathogenicity, in spite of showing marked differences in haemolysin production *in vitro*. Table 2 shows some characteristics of strains of *Staph. aureus* isolated from natural cases of mastitis. It can be seen that the degree of infectivity and pathogenicity are not necessarily correlated with α-haemolysin titre, nor with the numbers of flocculation lines formed.

We have examined the deoxyribonuclease (DNase) activity of staphylococci of bovine origin as Burns & Holtman (1961) considered that this property was closely correlated with virulence and coagulase production. Table 3 shows the distribution of coagulase and DNase activities in strains of staphylococci isolated from single herd and market milks. A comparison is also given with some of the findings of Burns & Holtman (1961). Coagulase and DNase are not completely correlated in either case, a number of DNase positive strains being coagulase negative.

Table 3. *A comparison of the coagulase and deoxyribonuclease (DNase) activities of staphylococci isolated from milk and from human sources*

Source of cultures	Incidence (% of isolates) of activity pattern*			
	(a)	(b)	(c)	(d)
Single herd and bulk milk (11 samples yielding 513 cultures)	57·3	30·6	1·4	10·7
Human clinical, nasal and skin isolates; hospital air; (Data of Burns & Holtman, 1961)	42·8	47·4	3·2	6·6

* Activity pattern: (a), coagulase and DNase positive; (b), coagulase and DNase negative; (c), coagulase positive, DNase negative; (d) coagulase negative, DNase positive.

(c) *Effect on yield and composition of milk*

Mastitis infections represent a serious loss to the farmer because of the deterioration in yield and composition of the milk.

The latest published figures, by Rowland, Neave, Dodd & Oliver (1959) and O'Donovan, Dodd & Neave (1960), show that the milk yield of affected quarters was reduced by an average of 15%, the solids-not-fat percentage by 0·20 and the fat percentage by 0·19. There was also a depression of 10–12% on total lactation yield of milk, fat and solids-not-fat. This occurred even though all infections giving rise to mastitis were treated at once with antibiotics.

(d) *Antibiotic treatment*

The results of antibiotic treatment of staphylococcal mastitis are not entirely encouraging. Antibiotics have generally failed to have any prolonged effect on the incidence of udder infection. Clinical response to treatment is often good but elimination of the infection probably does not occur in more than 50% of cases treated today, and relapses occur frequently. Also, unfortunately, some strains of both *Staph. aureus* and coagulase negative staphylococci produce penicillinase and the number of resistant strains of *Staph. aureus* isolated from mastitis has increased very considerably in the last few years, as has the number of strains relatively resistant to streptomycin and most other antibiotics. It seems unlikely that the new penicillins and other antibiotics will greatly increase the percentage of bacteriological cures of cases of mastitis unless abscess formation in the udder can be prevented or new preparations evolved that will penetrate these abscesses.

Many observers believe that staphylococcal mastitis has increased with the increase in machine milking, but the evidence for this is inconclusive. The marked reduction, through the use of penicillin, of the more infectious streptococci, which usually replaced the staphylococci in mixed infections, may have resulted in the increase in staphylococcal mastitis. On the other hand, erosion of the teat canal is associated with machine milking and in turn these erosions are almost invariably infected with staphylococci, so that milk samples will contain more staphylococcal whether or not there is an intramammary infection.

(e) *Transmission of staphylococci*

Although occasionally human carriers can transmit staphylococci to a healthy udder, the main source of infection is from an infected udder, and normal milking methods ensure their transfer from cow to cow. To reduce the number of infections strict milking hygiene precautions are necessary. Experiments at the Institute have shown that under these conditions cross infections are infrequent (Report, 1958*b*).

3. COAGULASE POSITIVE STAPHYLOCOCCI IN DAIRY PRODUCTS

Under favourable conditions, coagulase positive staphylococci present in raw milk may multiply excessively, either in the milk itself or during processing into cheese. If a toxin producing strain is present the enterotoxin formed may be sufficient to cause food poisoning.

(a) *Staphylococcal food poisoning*

There is good reason to believe that the most common form of food poisoning is due to staphylococci. The first association of staphylococci with food poisoning is usually attributed to Denys (1894). Nevertheless as recently as 1950 van Heyningen stated that progress in research on food poisoning due to staphylococcal enterotoxin is stifled almost to the point of extinction by the difficulty of testing for toxin. Since then some considerable progress has been made and the toxin has now been reasonably well purified and its antigenicity proven (Bergdoll, 1962). However, the toxin has either to be tested on animals (cats and monkeys) or on human volunteers. As long as routine laboratories have no means of testing contaminated food for toxin, the incrimination of the suspected food can only be substantiated by circumstantial evidence. Ethically the presence of large numbers of staphylococci in any foodstuff is to be considered undesirable and furthermore, as pointed out by Thatcher & Ross (1960), the dissemination into the community of large populations of virulent strains of staphylococci must be regarded as a public health hazard. A recent review of food borne epidemics due to staphylococci, with special reference to dairy products (Munch-Petersen, 1960), containing an extensive bibliography on the subject, stresses the fact that the incidence of staphylococci in man and bovine mastitis has increased of late.

(b) *Occurrence in milk and in cheese*

Raw milk may contain large numbers of staphylococci and as Jones, King, Fennel & Stone (1957) have shown, only rapid and efficient cooling on the farm with adequate heat treatment in the creamery (HTST) can safeguard the liquid milk and cream supply. Traditionally, however, cheese has been made from raw milk, and heat treatment of the manufacturing milk is usually aimed only at reducing the incidence of faults in the flavour, body and texture of the cheese due to contaminating bacteria. Heat treatment in this country varies from 145°F 'holding' treatment to 161°F 'flash' (from 7–22 sec) and the incidence of staphylococci in cheese could therefore be high, although no figures are as yet available. In Canada, Thatcher, Comtois, Ross & Erdman (1959) found that cheese made from raw milk frequently contained coagulase positive staphylococci with phage patterns commonly associated with human infections and cattle. Forty-five percent of Canadian cheese made from raw milk, and 13% of cheese made from heat treated milk, contained more than 10^5 coagulase positive staphylococci/g. No staphylococci were found among 236 imported cheeses usually made from pasteurized milk or heat processed after manufacture. The same authors demonstrated the presence of toxin in 8 out of 149 cheese samples taken from individual vats of cheese made in a factory selected because its products were highly contaminated with staphylococci. Similar experiences could be cited from other countries and it is of some interest that since the recent investigations of Jensen & Pedersen (1960) in Denmark on the heat resistance of some pathogenic bacteria occurring in milk and cheese, steps for adequate heat treatment of all cheesemilk appear to have been generally adopted, with subsequent freedom from *Staph. aureus* in all Danish cheese.

Experimental work on the multiplication of *Staph. aureus* in cheese (Mattick, Neave & Chapman, 1959; Takahashi & Johns, 1959; Thatcher & Ross, 1960; Roughley & McLeod, 1961—to name only a few of the more recent investigations) have shown that the numbers of staphylococci immediately increase during rennetting of the milk, because most of the cells are trapped in the curd in the same way as the starter (lactic acid streptococci). Takahashi & Johns (1959) actually worked out a formula for the calculation of the rate of multiplication, but this may in fact be immaterial; toxin is produced during multiplication of the organism and therefore the initial mechanical concentration in the curd is largely irrelevant. The temperatures during the cheesemaking process (84° to over 100°F) are favourable for growth and varying times between 4 and 6 or more hours are required until the salted or unsalted curd can be hooped in moulds and put to press. Takahashi & Johns (1959) found that the staphylococci increased to a greater extent when milk of high hygienic quality (low standard plate count) was made into cheese than when low grade milk was used. The contaminating flora may of course be potentially capable either of stimulating or inhibiting the staphylococci, depending on the predominant species in the milk. The same authors also stated that flash heating at 150°F appeared to have destroyed all staphylococci naturally present in the milk. It is generally agreed that the staphylococci die out rapidly during maturation of the cheese. Mattick *et al.* (1959) found that even small differences in pH (0·1–0·3) significantly affected the decline in numbers, the least rapid being in the sweeter cheese. The pH of the cheese, according to its kind and type, must therefore be of the greatest importance for the survival of the staphylococci. It is, however, clear that the enterotoxin is not similarly affected by time and remains active in the cheese for many months (Miljkovic, 1960). Thus the number of staphylococci cannot be a true indication of the presence or absence of enterotoxin, unless one takes into account the pH and age of the cheese.

(c) *Experimental work on the multiplication of* Staph. aureus *during cheesemaking*

Investigations undertaken at this Institute on some aspects of *Staph. aureus* in the manufacture and maturation of Cheddar cheese are described below. As the coagulase positive staphylococci are generally believed to be toxigenic a comparison was made in the first place of the selectivity of various media to decide which would be the most suitable for a survey of the incidence of coagulase positive staphylococci in raw milk and cheese throughout the country.

(i) *Selective media for isolation.*

Three different selective media were compared. One was a simplified form (CM) of the mannitol-salt medium of Chapman (1946); the salt content was reduced to 6·5% to avoid inhibitory phenomena and the mannitol was omitted to prevent the growth of certain lactobacilli occurring in milk and cheese which are capable of utilizing mannitol and are sufficiently salt tolerant to grow in 6·5% salt. The other media were the phenolphthalein phosphate medium (BK) of Barber & Kuper (1951), the use of which is based on a correlation between the phosphatase and coagulase reactions, and the tellurite-egg yolk medium (BP) of Baird-Parker (1962), which is both selective and diagnostic for coagulase positive staphylococci.

Samples of single herd and market milks were plated on the three media and representative numbers of colonies were tested for coagulase. The CM medium allowed the growth of both coagulase positive and negative staphylococci, 49·9% of the total number of colonies examined from all samples being coagulase positive. From the BK medium, which was nonselective, only phosphatase positive colonies were examined, 54·9% of these being coagulase positive. This lack of correlation between the phosphatase and coagulase test, indicated by the presence of so many phosphatase positive strains which were coagulase negative, was also found by Thatcher & Simon (1956). The Baird-Parker medium, however, was more satisfactory, as 85·2% of the colonies diagnosed as coagulase positive proved to be so.

An analysis of variance indicated that the proportions of coagulase positive bacteria obtained from the CM and BK media were not significantly different from each other, but were both significantly different from that given by the BP medium (the probability of such a result occurring by chance was less than 1 in 1,000). The highest percentages of coagulase positive organisms were detected on the BP medium and when an experienced worker enumerated the colonies showing typical diagnostic features it was seldom necessary to confirm the results by the coagulase test. A comparison of the numbers of coagulase positive staphylococci present on the three media showed little difference between the numbers occurring on the CM and BP media, although the numbers were sometimes slightly higher on the former. However, fewer coagulase positive colonies were often found on the BK medium, probably because it was nonselective and inhibition by other organisms occurred. The superiority of the BP medium was further stressed when samples of 9 cheeses were plated for viable staphylococci (Fig. 1). The difference between the media increased further with the age of the cheese, when the number of staphylococci had greatly decreased, probably because attenuated cells in the older cheeses were stimulated to grow on the more nutrient BP medium. The BP medium is therefore the medium of choice and in our opinion its advantages, in giving higher viable counts in some circumstances plus a high percentage of coagulase positive staphylococci, outweigh the disadvantage of its somewhat cumbersome preparation.

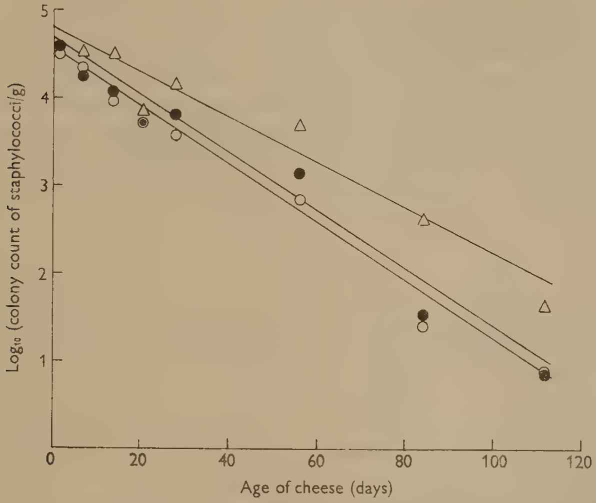

Fig. 1. A comparison of three different media for the enumeration of staphylococci in cheese. Triangles, Baird-Parker (1962) medium; open circles, Barber & Kuper (1951) medium; closed circles, modified Chapman (1946) medium. The lines have been fitted by regression analysis and best represent the observed data.

(ii) *Growth in milk.*

In preliminary experiments a suitable strain of *Staph. aureus* (which grew adequately in milk without any nutrient additives) isolated from a cow's udder and of phage type 42D was used. The strain grew in raw or pasteurized milk at a slower rate than in steamed or autoclaved milk. This was due to agglutination of the cells which interfered with the counts on agar, thus confirming the findings of Smith (1957) that milk contains 'nonspecific' agglutinins. In this work, whey agglutinin titres of up to 1/30th were found. Besides this inherent inaccuracy of staphylococcal counts in raw or insufficiently heat treated milk, their growth is also impaired by high inocula of lactic acid streptococci (1–2%) as used in cheesemaking. This is partly due to competition between the organisms (Jones *et al.* 1957), and partly to the rapid fall in pH caused by the acid producing streptococci. It was previously mentioned that the staphylococci die out rapidly at a pH of about 5 in cheese. This also occurs in other environments as was shown by Reiter, Vazquez & Newland (1961), who found that coagulase negative staphylococci died out overnight at low pH when grown in milk in symbiosis with lactic acid streptococci and/or lactobacilli.

(iii) *Cheesemaking conditions affecting multiplication.*

It was decided to make staphylococcus infected experimental cheese at significantly different pHs. Pairs of cheeses were made in separate vats from milk (30 gal each) heated to 161°F for 15 sec to eliminate any infecting staphylococci. Standard suspensions of a strain of *Staph. aureus* phage type 42D and starter (1%) were added to each vat. One vat of milk was also inoculated with sufficient homologous phage (10^3 phage particles/ml) to kill the starter during the cheesemaking and thus produce a 'slow' or 'sweet' cheese of high pH. Phage was chosen to kill the starter instead of antibiotics, because of the obvious complications which could arise from the effect of the antibiotic on the staphylococci. Both phage and antibiotics are known to affect the cheese quality by slowing down or killing the starter. Fig. 2 shows the results of a typical experiment. The staphylococcal counts in the 'phaged' cheese were appreciably higher at milling of the curd, which is the end of the actual cheesemaking process; they increased sharply in the first few days and more gradually later during maturation. The 'normal' cheese at a pH of about 5 showed an immediate decline of staphylococci after milling. During 6 months of maturation the staphylococci remained more or less stationary at a high level in the cheese of high pH. In another experiment when the pH fell much quicker in a phaged cheese, due to phage resistant streptococcus mutants producing acid during maturation, the staphylococcus count did not remain stationary but slowly declined.

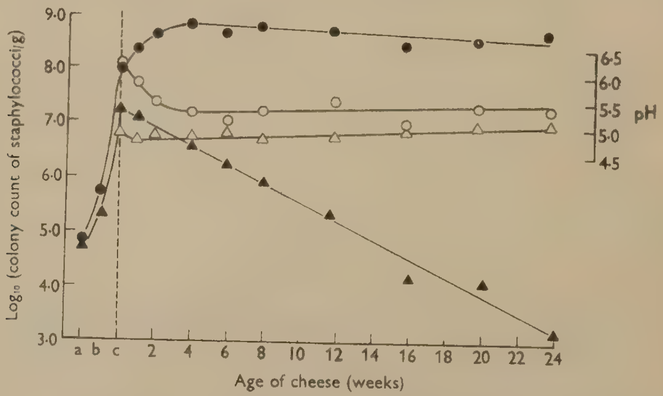

Fig. 2. The effect of starter failure due to bacteriophage on the multiplication of staphylococci during the making and maturation of Cheddar cheese. Closed circles, counts in slow cheese; closed triangles, counts in control cheese; open circles, pH of slow cheese; open triangles, pH of control cheese. Stages in cheese making: a, inoculation of milk; b, cutting of the curd; c, milling of the curd.

Roughley & McLeod (1961) found the same kind of decline of staphylococci in Cheddar cheese and they later (McLeod, Roughley & Richards, 1962) reported the failure of staphylococci to die out in a cheese of an unusually high pH (5·5–5·8), which was probably due to accidental phage or antibiotic contamination of the cheese-milk affecting the acid production of the starter.

It is of some interest to speculate whether the persistence of the staphylococci in the 'sweet' cheese is merely due to the survival of the organisms or whether some cells die out and are constantly replaced by new ones. In this latter case more enterotoxin might be produced.

These results emphasize that, until the enterotoxin in cheese can be assayed, viable counts of coagulase positive staphylococci provide little information. The age of the cheese and its pH must be taken into account to assess the potential danger for the consumer. Slow or 'sweet' cheeses should be regarded as suspect, unless proven free from staphylococci. In this connection we have examined a number of first and second grade imported cheeses and found that coagulase positive staphylococci were absent, or present in only small numbers, in first grade cheeses but very large numbers were sometimes found in second grade cheese.

4. COAGULASE NEGATIVE STAPHYLOCOCCI

In conclusion it may be of some interest that coagulase negative staphylococci possibly form an important part of the lipolytic flora of Cheddar cheese (Franklin & Sharpe, unpublished data). Alford & Frazier (1950) and Robertson & Perry (1961) have actually shown some enhancement in the flavour of Cheddar cheese by inoculating such strains into the cheesemilk. But as there is a suspicion that some coagulase negative staphylococci may produce enterotoxin (Thatcher & Simon, 1956) further investigations on the suitability of the addition of such organisms are warranted. This applies also to the penicillinase producing strains which have been used (Reiter *et al.* 1961; Vazquez & Reiter, 1962) to inactivate penicillin in cheese and yogurt milk inoculated with normal starter.

Whereas it is true that we do not know the conditions which promote the production of enterotoxin and that little progress can be made until reliable and simple tests are available for detecting and measuring it, the solution to the problem of protecting the consumer against toxic milk, cream or cheese is simply to use refrigeration and pasteurization.

5. SUMMARY

The staphylococci and micrococci which are frequently present in raw milk are derived from infected cows and from dairy equipment. Coagulase positive and coagulase negative staphylococci often occur in the cow's udder; the former usually give rise to mastitis and are now the commonest cause of this condition. Methods of isolation and some characteristics of such strains are described. The elimination of these organisms, which are often resistant to antibiotic treatment, is difficult but the use of strictly hygienic milking methods promises a decrease in the incidence of infection.

A comparison of selective media for isolating coagulase positive staphylococci from cheese favoured a tellurite-glycine-egg yolk medium. Excessive multiplication during

cheesemaking of the coagulase positive staphylococci present in raw or insufficiently heat treated milk may result in large numbers of these organisms, which decrease rapidly in normal cheese of low pH: as enterotoxin may remain active after the staphylococci have died out, the number of these organisms is not an indication of the presence or absence of toxin. Cheesemaking experiments showed that higher numbers of staphylococci were reached in 'sweet' cheese (having a high pH due to suppression of starter by bacteriophage) and no decline occurred after 6 months' ripening.

Lipolytic coagulase negative staphylococci in cheese may contribute towards the flavour, and penicillinase producing strains may be used to inactivate penicillin present in milk used for cheese and yogurt making.

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7. REFERENCES

ABD-EL-MALEK, Y. & GIBSON, T. (1948). Studies in the bacteriology of milk. II. The staphylococci and micrococci of milk. *J. Dairy Res.* **15**, 249.

ALFORD, J. A. & FRAZIER, W. C. (1950). Occurrence of micrococci in Cheddar cheese made from raw and pasteurized milk. *J. Dairy Sci.* **33**, 107.

ALLEN, V. D. & STOVALL, W. D. (1960). Laboratory aspects of staphylococcal food poisoning from Colby cheese. *Amer. J. publ. Hlth* **50**, 880.

BAIRD-PARKER, A. C. (1962). An improved diagnostic and selective medium for isolating coagulase positive staphylococci. *J. appl. Bact.* **25**, 12.

BARBER, M. & KUPER, S. W. A. (1951). Identification of *Staphylococcus pyogenes* by the phosphatase test. *J. Path. Bact.* **63**, 65.

BERGDOLL, M. S. (1962). Chemistry and detection of staphylococcal enterotoxin. In *Proc. 14th Res. Conf. Amer. Meat Inst. Found.*, p. 47. Chicago: Amer. Meat Inst. Found.

BURNS, J. & HOLTMAN, D. F. (1961). Biochemical properties of virulent and avirulent staphylococci. *Ann. N.Y. Acad. Sci.* **88**, 1115.

CHAPMAN, G. H. (1946). A single culture medium for selective isolation of plasma-coagulating staphylococci and for improved testing for chromogenesis, plasma coagulation, mannitol fermentation, and the Stone reaction. *J. Bact.* **51**, 409.

DENYS, J. (1894). Empoisonnement par de la viande contenant le staphylocoque pyogene. *Sem. Medicale* **14**, 441.

GARVIE, E. I. & CHEESEMAN, G. C. (1958). *Alcaligenes haemolysans*. *Nature, Lond.*, **181**, 1540.

GARVIE, E. I., HIGGS, T. M. & NEAVE, F. K. (1961). Unusual varieties of *Staphylococcus aureus*. *J. Path. Bact.* **81**, 255.

HAUSLER, W. J., BYERS, E. J., SCARBOROUGH, L. C. & HENDRICKS, S. L. (1960). Staphylococcal food intoxication due to Cheddar cheese. II. Laboratory evaluation. *J. Milk Fd Tech.* **23**, 1.

JENSEN, H. & PEDERSEN, A. H. (1960). Nogle patogene bakteriers termoresistens ved pasteurisering af ostemaelk og disse bakteriers resistens ved fremstilling og lagring af dansk ost. *Beretn. Forsøgslab. Kbh.* **124**, 31.

JONES, A. C., KING, G. J. G., FENNELL, H. & STONE, D. (1957). The growth of *Staph. aureus* in milk with special reference to food poisoning. *Mon. Bull. Ministr. Hlth Lab. Serv.* **16**, 109.

MCEWEN, A. D. & COOPER, M. B. (1947). Bovine mastitis. *Vet. Rec.* **59**, 655.

MCLEOD, R. W., ROUGHLEY, R. T. & RICHARDS, T. (1962). *Staphylococcus aureus* in Cheddar cheese. *Aust. J. Dairy Tech.* **17**, 154.

Mattick, A. T. R., Neave, F. K. & Chapman, H. R. (1959). *Staphylococcus aureus* in Cheddar cheese. *Int. Dairy Congr., Lond.*, **3**, 1914.

Miljkovic, K. ([illegible]). Die Erhaltung von Staphylokokken-enterotoxin in weissen Lake-Kase. *Arch. Lebensmittel Hyg.* **11**, (5), 20.

Munch-Petersen E. (1960). Food-borne epidemics due to staphylococci. *Aust. J. Dairy Tech.* **15**, 25.

O'Donovan, J., Dodd, F. H. & Neave, F. K. (1960). The effect of udder infections on the lactation yield of milk and milk solids. *J. Dairy Res.* **27**, 115.

Parker, M. T., Tomlinson, A. J. H. & Williams, R. E. O. (1955). Impetigo contagiosa. The association of certain types of *Staphylococcus aureus* and of *Streptococcus pyogenes* with superficial skin infections. *J. Hyg., Camb.*, **53**, 458.

Reiter, B., Vazquez, D. & Newland, L. G. M. (1961). The effect of the presence of penicillinase-producing micrococci on the growth of cheese and yogurt starters in milk contaminated with penicillin. *J. Dairy Res.* **28**, 183.

Report (1958*a*). Food poisoning in England and Wales, 1958. A Report of the Public Health Laboratory Service. *Mon. Bull. Ministr. Hlth Lab. Serv.* **18**, 169.

Report (1958*b*). *Rep. Nat. Inst. Res. Dairying, Reading*, 1958, p. 66.

Robertson, P. S. & Perry, K. D. (1961). Enhancement of the flavour of Cheddar cheese by adding a strain of micrococcus to the milk. *J. Dairy Res.* **28**, 245.

Roughley, R. J. & McLeod, R. W. (1961). Longevity of *Staphylococcus aureus* in Australian Cheddar cheese. *Aust. J. Dairy Tech.* **16**, 110.

Rowland, S. J., Neave, F. K., Dodd, F. H. & Oliver, J. (1959). The effect of *Staphylococcus pyogenes* infections on milk secretion. *Int. Dairy Congr., Lond.*, **1**, 121.

Shaw, C., Stitt, J. M. & Cowan, S. T. (1951). Staphylococci and their classification. *J. gen. Microbiol.* **5**, 1010.

Smith, H. W. (1948). The typing of staphylococci of animal origin by the bacteriophage method. *J. comp. Path.* **58**, 179.

Smith, H. W. (1954). The pathogenicity and haemolytic properties of staphylococci isolated from chickens. *J. Path. Bact.* **67**, 73.

Smith, H. W. (1957). The multiplication of *Staphylococcus aureus* in cows' milk. *Mon. Bull. Ministr. Hlth Lab. Serv.* **16**, 39.

Smith, J. (1961). Reviews of the progress of dairy science. *J. Dairy Res.* **28**, 87.

Takahashi, I. & Johns, C. K. (1959). *Staphylococcus aureus* in Cheddar cheese. *J. Dairy Sci.* **42**, 1032.

Thatcher, F. S. & Ross, D. (1960). Multiplication of staphylococci during cheesemaking: the influence of milk storage temperatures and of antibiotics. *Canad. J. publ. Hlth* **51**, 226.

Thatcher, F. S. & Simon, W. (1956). A comparative appraisal of the properties of staphylococci isolated from clinical sites and from dairy products. *Canad. J. Microbiol.* **2**, 703.

Thatcher, F. S., Comtois, R. D., Ross, D. & Erdman, I. E. (1959). Staphylococci in cheese: some public health aspects. *Canad. J. publ. Hlth* **50**, 497.

van Heyningen, W. E. (1950). *Bacterial Toxins.* Oxford: Blackwell Scientific Publications.

Vazquez, D. & Reiter, B. (1962). Making yogurt from penicillin contaminated milk. *Dairy Ind.* **27**, 525.

(*Received* 23 *August*, 1962)

Kitchell, A. G. (1962). *J. appl. Bact.* 25 (3), 416–431.

(Symposium on Staphylococci and Micrococci: Paper X)

MICROCOCCI AND COAGULASE NEGATIVE STAPHYLOCOCCI IN CURED MEATS AND MEAT PRODUCTS

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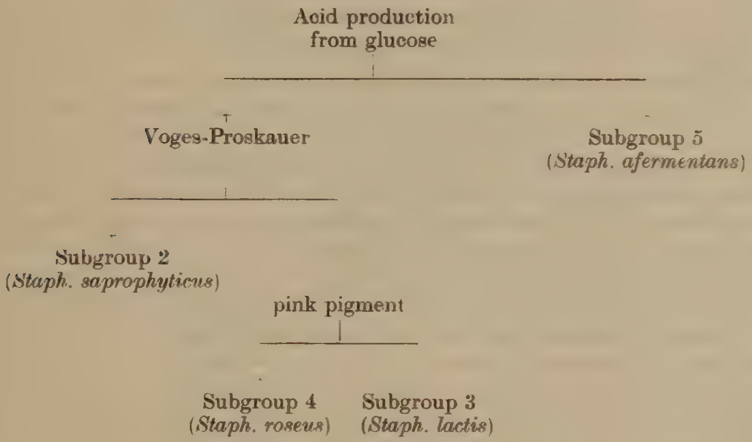
1. Introduction

Two major difficulties have been encountered in the writing of this review. The first is the paucity of specific information in the literature on the numbers, proportions, and types of micrococci occurring in cured meats and meat products and their activity in these foods. The second concerns nomenclature.

Having obtained data from early work, it was frequently impossible to correlate them with later findings because of changes in classification and nomenclature. For example, the catalase positive, Gram-positive cocci, whether fermentative, oxidative or toxigenic, have on the one hand been assigned to the genera *Micrococcus*, *Gaffkya*, and *Sarcina* (Breed, Murray & Hitchens, 1948) and on the other hand to the single genus *Staphylococcus* (Shaw, Stitt & Cowan, 1951). Descriptions both in the literature and in the earlier editions of Bergey's *Manual* are often not sufficiently detailed to allow organisms to be renamed according to modern nomenclature.

Further, experience with the use of the key set out in Bergey's *Manual* proved that it was not helpful in sorting micrococci from pork, bacon, and bacon brines, very many of which failed to fit the described species. The classification of Shaw *et al.* (1951) was more useful in separating them into a few well defined groups (Kitchell, 1958). Four subgroups within the Gram-positive, catalase positive, coagulase negative cocci were recognized on the basis of pink pigmentation and only two tests, viz. acid production from glucose and the Voges-Proskauer reaction.



Since, for the purpose of this paper, it is convenient to distinguish between micrococci and coagulase negative staphylococci it has been decided to do this on the basis of the above subgroups. Following indications from the work of Evans, Bradford & Niven (1955) that the fermentative metabolism of glucose, which characterizes members of the genus *Staphylococcus*, was restricted to subgroups 1 and 2 and that members of the other subgroups of Shaw *et al.* (1951) utilized glucose oxidatively or not at all, it is proposed to use the term micrococcus for the latter and to describe the organisms in subgroup 2 as coagulase negative staphylococci. Coagulase positive organisms (subgroup 1) are specifically excluded from consideration in this review.

In quoting from the older literature, especially quantitative data, all isolates belonging to the Micrococcaceae, no matter how described by the author, have been treated collectively and named micrococci. The distinction drawn above between micrococci and coagulase negative staphylococci is relevant mainly in relation to more recent work on bacon.

Baird-Parker (1963) has now shown that splitting staphylococci from micrococci on the basis of fermentative as opposed to oxidative breakdown of glucose requires reorganizations to be made within subgroups 2 and 3 of Shaw *et al.* (1951). However, results obtained by him on the composition of the microflora of bacon (Baird-Parker,

1962) are not dissimilar from those of Kitchell (1958 and unpublished data) using the scheme of Shaw *et al.* (1951).

Simple classifications such as that of Shaw *et al.* (1951) may have limitations from the taxonomic point of view. Nevertheless, without some such ready means of characterizing his isolates, a meat bacteriologist finds it impossible to recognize specific contaminants and thereby determine their frequency of occurrence, relative numbers, origin and function.

2. The Relative Numbers and Frequency of Occurrence of Micrococci

Before surveying the reports in the literature of the occurrence of micrococci in various cured meats and meat products, some of the activities of these bacteria which may be of importance either in production or in relation to storage stability should be reviewed briefly. The subject will be treated in detail later but an introduction to the topics now, with emphasis on the influence on bacterial activity of temperature and curing ingredients, will make it unnecessary to make these points as the findings of each investigator are described.

Cold tolerant and salt tolerant strains of micrococci are of the utmost importance in cured meats which depend for their stability on the inhibitory action of NaCl and storage at refrigerator temperatures. Their occurrence on the carcase meat from which cured products are made is of interest in establishing the level of initial contamination.

There is also an effect of higher temperatures to be considered. For example, vacuum packed sliced meats, having an appearance not unlike that of fully processed products, are often abused by storage at ambient temperatures. Under these conditions the type of organism and the nature of the spoilage may be different from those observed at lower temperatures.

The desirable chemical changes brought about by micrococci in fermented sausages, for example, are affected by both temperature and salt concentration. It is not altogether clear to what particular activities beneficial or deleterious changes may be attributed. However, it seems likely that the reduction of nitrate to nitrite, production of acid from carbohydrates, proteolysis, lipolysis and the oxidation of fats are factors of importance. Also, the fact that nitrate can serve as an alternative hydrogen acceptor to atmospheric oxygen means that 'aerobic' micrococci are able to grow within meat products and on vacuum packed meats in what would generally be regarded as anaerobic environments.

(a) *Carcase meat: uncured*

Beef. The principal sources of contamination of carcase meat in the abattoir are soil, water, air and the gut of the slaughtered animal (Haines, 1933*a*). In various places about the slaughterhouse, Haines isolated 18 different species of micrococci and estimated that they constituted 46–47% of the microflora of the air. Examination of the slime developing on small joints of beef stored under refrigeration (Haines, 1933*b*) showed that micrococci accounted for 20–40% of the bacteria recovered from plates incubated at 37°. At 20° and 4°, Gram-negative rods predominated and no micrococci were found. Also, at these temperatures the bacterial counts were a thousand times greater than at 37°.

Examination of the surface of freshly slaughtered beef (Empey & Scott, 1939) showed that, of the bacteria growing at 20°, approximately 50% were micrococci. That most of these were not cold tolerant types, or could not compete with other organisms at low temperatures, became clear when these workers determined the composition of the microflora able to grow at −1°. Micrococci represented only 7%, the balance consisting of '*Achromobacter*' (90%), *Flavobacterium* (3%), and *Pseudomonas* (less than 1%). Thus the typical spoilage microflora of chilled beef is dominated by Gram-negative rods. It should be noted, however, that when Moran & Smith (1929) examined beef hung for 15 days at 5° (70% R.H.) they found that 71% of the microflora belonged to two species of micrococci.

Pork. Jepsen (1947) investigated a slaughterhouse handling pigs and made isolations from the carcase at all stages of the slaughter floor treatment. Of 239 aerobic and facultatively anaerobic strains isolated at 25° or 2°, 39% were micrococci. Examination of these 94 strains showed that 74% reduced nitrate, 53% produced acid from glucose and 40% liquefied gelatin. When 52 of these micrococci were tested for growth at 2°–3°, 73% were positive. Of 29 strains tested, 72% grew at 25° in a medium containing 10% of NaCl. Only 4 of the 29 strains belonged to the 52 selected for cold tolerance tests and of these only 2 were positive in both tests.

In spite of this indication of the presence of a fair proportion of cold tolerant micrococci it is generally observed that pork, like beef, is spoiled by a predominantly Gram-negative microflora. It would be of interest to know why this is so.

(b) *Carcase meat: cured*

The products made by curing portions of the carcase are chiefly salt beef, salt pork, sides of Wiltshire bacon and hams. Curing may be effected either by immersion in a brine or by dry salting, though the former method is much more common. The only dry cured product to which reference will be made is charque (salt beef).

Brines. The bacteriology of meat curing brines, which may contain up to 26% NaCl, is a very specialized field. Many brines contain large numbers of micrococci, often as the predominant organism, but many do not and appear to be equally satisfactory in use. The subject is dealt with in detail in the Proceedings of the 2nd International Symposium on Food Microbiology (Proceedings, 1958) and by Leistner (1958*a*,*c*, 1959) and it is not proposed to discuss it here.

Dry cure. Dry cured beef (charque) develops a superficial slime before drying (Gutheil, 1958) which consists chiefly of micrococci. It is believed that a moderate growth of these bacteria is essential for the development of the characteristic flavour of the dried meat.

Brine cure. Sides of pork going into pickle have a microflora of which about 40% are micrococci (Garrard & Lochhead, 1939, and cf. Jepsen, 1947). Ten different types were recognized and of these 6 reduced nitrate, 8 produced acid from glucose, 5 liquefied gelatin, and 6 were lipolytic. All grew in 10% NaCl and only one was able to do so at 25%. After curing and during maturation at 5°, a superficial bacterial slime develops. In the case of bacon (Kitchell, 1958) at least half of the organisms are micrococci belonging to subgroup 3 (*Staph. lactis*) of Shaw *et al*. (1951). Fifty five isolates were examined and it was found that 98% produced acid from glucose and 96% reduced nitrate.

It should be noted that the proportions of micrococci have been reported to be as high as 94% on sides of pork before curing and 89–100% on matured sides of bacon (Brooks, Haines, Moran & Pace, 1940).

Horovitz-Vlasova & Livshitz (1931) recognized that the predominant organism in the slime forming on salt pork was a lipolytic micrococcus. The types of bacteria found by Ingram (1934) in bacon slime were micrococci, lactic acid bacteria, and aerobic sporeformers.

Ingram (1952) distinguished between gammons, i.e. legs cut from normally cured Wiltshire sides which have been pumped with up to 5% of their weight of brine, and hams cured separately without pumping. In examining samples of each exhibiting internal spoilage ('taint' or 'souring') he found that the gammons yielded 16 species of micrococci presumed to have come from the pumping brine whereas only 4 species were found in the hams. The off flavours produced in gammons by salt tolerant micrococci tended to be cheesy and rancid rather than offensively putrid as in the hams.

(c) *Canned meats*

Canned cured meats and meat products are not all given the same level of heat processing. Some are fully processed to commercial 'sterility' but others, which would lose their characteristic qualities as a result of such a process, are pasteurized: these latter are the so-called semipreserved meats.

Micrococci have been associated with both types of product. For example, Skovgaard (1959) isolated micrococci from supposedly sterile canned luncheon meat and ham. He also found them in 9% of 287 cans of contaminated semipreserved ham, pork loin and Canadian bacon. They were present in 45% of 100 cans of sliced bacon.

The most important and frequently occurring group of bacteria isolated from defective pasteurized canned hams (Ingram & Hobbs, 1954) was that consisting of 19 species of micrococci. In a survey, mainly of corned beef, made by Savage, Hunwicke & Calder (1922), 17% of 86 unspoiled samples contained viable micrococci whereas 40% of 30 spoiled cans harboured these organisms. However, they were discounted by these workers as the cause of spoilage for a number of reasons, not least because inoculation of strains from spoiled cans into experimental cans produced no deleterious changes though growth occurred.

It remains to be explained, nonetheless, how it is that micrococci are found in heat processed canned meats. Ingram (1955) established that, in the presence of fat, the heat resistance of micrococci is such that it is likely that they would survive the pasteurizing process given to semipreserved products. This increased heat resistance is unlikely to explain their presence in a fully processed canned meat.

In these cases it must be assumed that contamination occurred, after processing, through leaks in the can or that the contaminating micrococci were introduced in the laboratory at the time of sampling.

(d) *Continental sausage products*

It is sufficient for the purpose of this paper to distinguish only two types of sausage, viz. cooked and raw. The former, represented by frankfurters, for example, are spoiled by the development of a superficial slime and it is in this connection that micrococci are of interest. Raw sausages (e.g. salami) are first fermented and then subjected to prolonged 'ripening' during which desiccation stabilizes the product. Smoking may also be part of the process.

Cooked sausages. Roderick & Norton (1936; cited by Ogilvy & Ayres, 1953) found that the slime which terminated the life of frankfurters consisted mainly of aerobic, rarely proteolytic, nonpigmented micrococci producing acid from sugars. More recent work (Ogilvy & Ayres, 1953) indicated that micrococci and bacilli were the most numerous Gram-positive bacteria on refrigerated frankfurters (4·4°–10°), together with smaller numbers of lactic acid bacteria and Gram-negative rods. Of the 57 micrococci studied by these workers 60% produced acid from glucose whereas another 25% gave equivocal results, 60% reduced nitrate and 60% were proteolytic (cf. Roderick & Norton, 1936). On the other hand, Evans & Niven (1955) found only 11% of micrococci in 353 isolations made from slime on frankfurters held at refrigerator temperatures down to 2°.

Liver sausage and bologna (Steinke & Foster, 1951*a,b,c*) also develop superficial slimes dominated by species of micrococci. At 30° and 16° the dominant species on liver sausage were *M. candidus* and *M. epidermidis*, neither of which grew on the product stored at 10·5° or 5°. Only *M. candidus* was found on bologna; but it persisted down to 10·5° though not to 5°.

Raw sausages. Early work in this field (Cesari, 1919) established the presence of yellow and white micrococci, yeasts, *Escherichia coli* and *Proteus* species in ripening sausage. Because the bacteria found were more normally associated with spoilage, Cesari concluded that the yeasts were likely to have the primary role in maturation, together with the micrococci. However, other genera, especially Gram-negative rods and lactic acid bacteria, have since been implicated (e.g. Jensen & Paddock, 1940; Politi, Colla & Corberi, 1944; Keller, 1954; Keller & Meyer, 1954; Zanzucchi & Delindati, 1956; Lerche, 1956; Coretti, 1956; Deibel & Niven, 1957).

The micrococci in these dry sausages have been the subject of special study by Niinivaara & Pohja (1957*a*) and Pohja (1960). As reported by other workers (e.g. Lerche, 1956; Coretti, 1956), they found that the mixed microflora of the fresh sausage emulsion becomes dominated, in the initial stages of manufacture before drying, by micrococci which disappear during the smoking process leaving mainly lactobacilli, a few cocci and Gram-negative rods. Working with 215 isolates they have identified 27 different species of bacteria which, using Bergey's nomenclature, include *M. aurantiacus*, *M. caseolyticus*, *M. canudids*, *M. conglomeratus*, *M. epidermidis* *M. flavus*, *M. luteus*, *M. ureae*, *M. varians*, *Gaffkya tetragena*, *Sarcina citrea*, *Sarcina* *lutea*, and white and yellow pigmented *M. pyogenes*. Eight of the species of micrococci reduced nitrate to nitrite and all except *G. tetragena* produced acid from glucose.

(e) *Prepacked cured meat products*

There are two main types of product and two forms of packing to be considered. Micrococci constitute an important part of the spoilage flora developing on processed cured meats (e.g. luncheon meats) or on sliced raw bacon. Both simple overwrapping with a transparent film (e.g. Cellophane) and vacuum packing in an oxygen impermeable film are used for these products.

Processed products. A study of the distribution of bacterial species among 50 random samples of each of seven types of luncheon meats prepacked in transparent films (Brooks & Henrickson, 1956) revealed that *M. ureae*, followed by *M. varians*, were most widely distributed in terms of type of product and frequency of isolation at 15° and 37°. *M. conglomeratus*, though not isolated from every product, was found in a larger number of samples than any other single species. About half the samples of each of 3 products were simply overwrapped with Cellophane, the other half being vacuum packed. Quantitative and qualitative changes in the microflora could not be related to the different methods of packing.

Alm, Erichsen & Molin (1961) also reported that micrococci predominated in Cellophane wrapped sliced cured meats. However, lactic acid bacteria predominated in vacuum packed products.

Bacon. The microflora of vacuum packed sliced bacon was found to contain 40% of micrococci when stored at 20° for 2 days (Hansen, 1960) although their numbers depend on temperature and in any case decrease with time of storage. Cavett (1962) also reported spoilage of vacuum packed bacon by micrococci and lactic acid bacteria. At 30°, the predominant organisms were coagulase negative, fermentative staphylococci whereas oxidative micrococci were most common at 20°.

In recent experiments (Kitchell, unpublished data) involving nearly 600 isolations made from sliced Wiltshire bacon, 41% were micrococci or coagulase negative staphylococci. After vacuum packing and storage at 5°, 20° and 30° until spoiled, the proportions of these types were found to be between 23 and 43%, the highest temperature of storage having the highest proportion, mainly due to the growth of subgroup 2 strains (Shaw *et al*. 1951). Lactic acid bacteria constituted most of the remainder of the microflora.

Much higher proportions of micrococci have been reported, e.g. 80% (Hankins, Sulzbacher, Kauffman & Mayo, 1950), but this was in sliced American bacon vacuum packed in cans. Only 4% of isolates were classified as staphylococci. The product was stored at 37° and isolations were made at that temperature. Under similar conditions Ingram (1960) found that all isolations made at 37° from sliced Wiltshire bacon stored at the same temperature were micrococci or coagulase negative staphylococci. After storage for 2 days in air, all but one of 14 strains examined were coagulase negative staphylococci. Of 22 strains isolated after 1 day of storage under 95% nitrogen and 5% oxygen, only 23% were coagulase negative staphylococci and the remainder were subgroup 3 types, i.e. *Staph. lactis*.

3. THE ACTIVITIES OF MICROCOCCI IN MEAT PRODUCTS

(a) *Nitrate reduction and colour production*

In cured meats the characteristic colour develops as a result of a chemical reaction between nitrite and the muscle pigment myoglobin, resulting in the formation of nitrosomyoglobin. Although nitrite itself may be used in curing processes (in some countries its use is prohibited). it is still the general practice to use nitrate. The curer is thus dependent on bacterial action to reduce nitrate to nitrite.

Nitrate reduction. It is in this connection that micrococci undoubtedly play a significant part in curing. Seventy-one percent of 402 strains examined by Shaw *et al.* (1951), 96% of 55 micrococci from bacon slime (Kitchell, 1958), 90% of 59 strains from bacon brines (Kitchell, 1958) and 84% of 171 micrococci from brines and dry sausages (Pohja, 1960) reduced nitrate to nitrite.

Of the 40 different bacterial types recognized by Garrard & Lochhead (1939) among 285 isolates from bacon pigs before slaughter, 26 reduced nitrate. These types, which included 10 micrococci, were studied with respect to the influence of NaCl concentration on both growth and nitrate reduction. All strains grew at concentrations of NaCl up to 10%. 15 grew at 15%, 8 at 20% and 3 at 25%. However, nitrate was reduced by all strains only up to 5% NaCl. 24 reduced nitrate at 10%, 11 at 15%, 4 at 20% and 1 at 25%. On the other hand. it has been reported (Brooks *et al.* 1940) that many nonhalophilic cocci from bacon brine continued to reduce nitrate to some extent even in 25% NaCl.

Eddy (1958) studied a nitrate reducing micrococcus isolated from bacon brine. Nitratase activity at 37° was more than tripled by the incorporation of 15% of NaCl in the growth medium. though nitrate was without effect. In the presence of 20% of NaCl the activity of cells grown in 15% salt was one-third of that in 3% NaCl. In the range 10° to 30°, nitratase activity in 3% NaCl roughly doubled for every 10° rise of temperature.

It is known that in sides of bacon (Eddy, Gatherum & Kitchell, 1960) not all of the nitrate reduced remains in the meat as nitrite. There are strong indications from model experiments that as much as two-thirds of the nitrite may be further reduced (to nitrogen?) by the bacteria present in the surface slime. Unfortunately, it is not known which organisms have this ability, mainly because a simple and satisfactory method for testing strains directly for nitrite reduction is not available. Such tests as have been made (Eddy. 1958, p. 95) indicated that 39% of 114 micrococci from pork, bacon and brine reduced nitrite.

In the experiments with packed bacon reported by Cavett (1962) it has been shown by Baird-Parker (1962) that it was the coagulase negative, fermentative staphylococci dominant at 30° which reduced nitrite and that the oxidative micrococci found chiefly at 20° reduced only nitrate. Although the greater disappearance of nitrite and the greater proportion of coagulase negative staphylococci at higher temperatures have been confirmed (Kitchell. unpublished data), we have no direct evidence to correlate these observations.

Colour. The formation of the cured meat pigment is much influenced by pH, proceeding faster under acid conditions. Thus organisms producing acid from glucose, a sugar present in about 0·9% concentration in pork muscle, as well as reducing nitrate are helpful in curing. For this reason Niinivaara (1955) recommended the use of a strain of micrococcus, M_{53}, resembling *M. aurantiacus*, as a starter in the production of dry sausage. A desirable acidity, pH 5·4, was reached within 48 hr instead of the 5 days required in normal manufacture. Good colour production was assured and, in addition, the starter culture was found to inhibit many undesirable contaminants normally found in the sausage mix. Of course, the ability to produce acid from glucose is common among meat micrococci (92% of all isolates—Kitchell, 1958). However, as has already been noted, micrococci are very frequently associated with lactic acid bacteria. In these circumstances, overproduction of acid with the attendant risk of pigment oxidation (Greenwood, Lewis, Urbain & Jensen, 1940) may well be as great a problem as underproduction.

(b) *Aroma and flavour production*

The claim is made by Pohja (1960) that micrococci are ". . . superior to the lactobacilli in their versatility as producers of desirable characteristics" and he goes on to refer to research on ". . . their contribution to the improvement of colour, aroma, flavour and keeping quality in so-called dry sausages." However, in recording their results, Niinivaara & Pohja (1957*a*,*b*) do not make specific reference to the eating qualities or aroma of sausages prepared with the starter culture M_{53}, although it is implied that they are satisfactory in all respects. Nevertheless, their results show clearly that other species are present; indeed lactic acid bacteria are said to be highly desirable and it is difficult to know which specific qualities of the product are attributable to micrococci.

The same workers have remarked on the agreeable aromas produced by Gram-negative rods, as have Keller (1954) and Zanzucchi & Delindati (1956). More recently, Lerche (pers. comm.) has concluded that the Gram-negative microflora plays no significant part in producing aroma and flavour in dry sausage, which he believes are the result of the action of micrococci, lactic acid bacteria and yeasts. In pure culture studies, Lerche found it best to use a mixture of these organisms, the most effective being a combination of micrococci with yeasts (cf. Cesari, 1919).

Thus the value of using specific bacteria for aroma and flavour production in Continental sausage products remains obscure. Regrettably, the same is true of bacon, there being no general agreement as to whether a process of maturation involving the activity of micro-organisms makes any contribution to flavour. Claims have been made for a role for micrococci (e.g. Leistner, 1958*b*) and Ingram has suggested that diacetyl, produced for example by coagulase negative staphylococci (Eddy, 1961) and lactic acid bacteria, may be involved.

(c) *Spoilage*

Dr. M. Ingram has often remarked that the smell of surface slime on sides of Wiltshire bacon is not at all like that found on fresh pork, which is both stronger and more unpleasant. The fact that the spoilage flora of pork is largely composed of Gram-negative bacteria is not necessarily the reason for this difference. It is clear that there can be at least as many Gram-negative bacteria as there are micrococci in bacon slime developing at 5° (Kitchell, 1958), although their nature is not known and they may be different from those on fresh pork.

However, another possible explanation would appear to be the influence of salt on the metabolism of the bacon spoilage bacteria, resulting in different end products. Unfortunately there is very little known about the chemistry of the spoilage of cured meats by specific types of bacteria.

Cavett (1962) has correlated the type of spoilage in vacuum packed bacon with the composition of the microflora. The spoilage odour at 30° was 'putrid', whereas at 20° it was 'scented-sour'. At the higher temperature, coagulase negative, fermentative staphylococci predominated but at 20° oxidative micrococci were found. In both cases the balance of the flora was mainly lactic acid bacteria.

Chemical analyses (Cavett, 1962; and data to be published) showed that whereas at 20° amino acids and free fatty acids appeared slowly, at 30° they appeared much more rapidly. It is suggested that rapid proteolysis caused by the staphylococci produced the 'putrid' odour and that the 'scented-sour' odour resulted from the liberation of free fatty acids by micrococci and the production of volatile substances by lactic acid bacteria.

Proteolysis. Proteolysis and lipolysis, quite common activities of many micrococci, are obvious chemical changes to associate with the spoilage of meat. Nevertheless, though 77% of 137 meat strains liquefied gelatin only 13% were active against pasteurized (80° for 10 min) meat protein (Pohja, Rigler & Niinivaara, 1960; Pohja & Niinivaara, 1961). Furthermore, these tests were made in the absence of NaCl. In view of the work of the same authors with *Bacillus* spp. (Pohja & Niinivaara, 1960) and that of Rockwell & Ebertz (1924), which established that the liquefaction of coagulated blood serum by *Proteus* spp. was inhibited by 4% of NaCl whereas 11% was required to halt growth, it is clearly essential to consider the influence of salt concentration on the course of proteolysis as such. Niinivaara (1962) believes that proteolytic bacteria have little chance of developing activity in dry sausage under the prevailing conditions of salt concentration and pH. Nevertheless, work by Dussault (1958) suggests that the ability of some micrococci from lightly salted fish to hydrolyze casein continues with decreasing vigour up to the maximum salt concentration permitting growth.

Ulrich & Halvorson (1951) found that the best chemical index of the proteolytic spoilage of canned sliced bacon stored at 38° was the ratio of ammonia-N to Kjeldahl-N. Also a rapid increase in ammonia-N could be correlated with bacterial growth at 38° but not at 4·4° (cf. Cavett, 1962, the effect of temperature on spoilage). Niinivaara & Pohja (1957*a*) attributed undesirable changes produced by certain bacteria in dry sausage to ammonia production. The aerobic spoilage of sides of Wiltshire bacon usually results in a rise in pH and experiments with cured pork fillets (*psoas* muscle) have shown this to be due to the activity of the surface microflora (Eddy *et al.* 1960). The fact remains, however, that this type of spoilage is not characterized by ammoniacal odours.

Rancidity. Spoilage changes in meat products which are associated with the fat rather than the lean are of two kinds, viz. hydrolytic or oxidative, which may or may not be enzymic. The hydrolytic breakdown to free fatty acids is commonly associated with the action of bacterial lipases. An example has already been cited above (Cavett, 1962). Though Ulrich & Halvorson (1951) observed in sliced bacon the steady hydrolysis of fat during storage, as reported by White (1941) and Jensen (1954), they were unable to correlate it with the population of lipolytic bacteria. They attributed it to the action of pork tissue lipases. Increasing the NaCl concentration was found to decrease the rate of hydrolysis.

As in tests for proteolysis, so with lipolysis it may be important to use a substrate related to the food being spoiled. Thus it would be interesting to know how many of Pohja's strains, 25% of which were positive when tested on arachis (ground nut) oil, would attack pork fat. If tests comparing tributyrin and lard are any indication, and they may not be, the difference is unlikely to be as great as between gelatin and meat protein as substrates for proteolytic tests. Of 223 micrococci from bacon and bacon brines, 54% split tributyrin and 38% split lard and tributyrin (Kitchell, unpublished data). In neither case was the influence of NaCl concentration determined and this is obviously of considerable practical importance.

The very fact of packing in an oxygen impermeable film or can under vacuum does much to give protection against the oxidation of fat, as was confirmed for bacon by Ulrich & Halvorson (1951). There is, however, evidence to suggest that organisms giving a positive result in an oxidase test with dimethyl or tetramethyl-*p*-phenylenediamine are capable of accelerating fat oxidation (Jensen, 1954). Fourteen percent of Pohja's (1960) 171 strains were oxidase positive, as were 78 (28%) of 277 micrococci isolated from various meats (Kitchell, unpublished data). Distribution of the 78 oxidase positive strains into subgroups indicated that 10% were in subgroup 2, 60% in subgroup 3 and 30% in subgroup 5.

Clearly, the factors governing the development of 'rancidity' in fat are complex. All that can be said of the role of micrococci is that a number of those isolated from meat products possess enzymes capable of bringing about relevant reactions.

Discolouration. Oxidative rancidity can result in the development of a yellow discolouration in bacon fat and this is accelerated under alkaline conditions. Thus the change already noted in the pH of bacon during spoilage may accentuate discolouration of the fat. Other and more lurid discolourations are described by Jensen (1954) and are attributed to the fat soluble carotenoid pigments of some micrococci.

There are difficulties in preserving the colour of the lean of vacuum packed bacon but bacteria are not generally implicated, except that they may offer some protection by consuming residual oxygen. There is, however, another aspect of this problem which may be worth noting. Sodium ascorbate has been recommended for use in cured meat products to accelerate the production of nitrosomyoglobin. It has also been suggested that spraying, sliced bacon for example, with sodium ascorbate will stabilize the colour. In practice, results with spraying have been disappointing because protection is short lived. Part of the reason for this may be that *M. varians*, a strain frequently reported in cured meats, is able to metabolize ascorbic acid to pyruvate, carbon dioxide and water (Kamiya, 1960; Kamiya, Yamamoto & Nakabayashi, 1960).

(d) *Antibacterial activity*

Strains of micrococci isolated from sea water have been shown to inhibit other bacterial species (Rosenfeld & ZoBell, 1947). Micrococcin, an antibacterial substance, is produced by a strain resembling *M. varians* (Su, 1948). Hawley (pers. comm.) is of the opinion that many of the micrococci from meat and meat products are capable of producing rather weak polypeptide antibiotics, some only in the presence of NaCl.

Niinivaara (1955, 1958) found that the starter strain M_{53} produced an antibiotic inhibitory to many of the miscellaneous and undesirable bacteria normally present in the raw sausage mix. Examination of 114 samples of sausage from 11 factories using the starter culture (Niinivaara & Pohja, 1957*b*) showed that there were many fewer defective products than were found in normal manufacture. Forty-one out of 63 samples analyzed in detail were found to contain mainly lactic acid bacteria and a few micrococci; these were the most acceptable and technically perfect sausages. The final composition of the microflora may be explained by the resistance of lactic acid bacteria to the antibiotic produced by M_{53} and the fact that micrococci nearly always disappear during drying and smoking (see below).

Apart from organic antibiotics, there are two other ways in which micrococci may inhibit not only other bacteria but also, possibly, micrococci themselves. Both are related to nitrogen metabolism.

Nitrite is toxic to many bacteria, especially under acidic conditions (Tarr, 1941). Species inhibited at 200 p/m (the maximum amount permitted to be added to cured meats) at pH 5·7 or 6·0 belonged to the genera *Achromobacter*, *Flavobacterium*, *Pseudomonas*, *Escherichia*, *Aerobacter* and *Micrococcus*. In dry sausage manufacture it seems a distinct possibility that nitrite inhibition accounts for the disappearance of micrococci. Deibel, Niven & Wilson (1961) established that during the heating and smoking process, after which lactic acid bacteria predominate, nitrate reduction occurs before acidification. Thus micrococci have played their part before acidification causes nitrite to become inhibitory.

In the further reduction of nitrite which has been observed in bacon it is not unlikely that hydroxylamine is an intermediate compound. This chemical also is toxic for a range of bacteria (Jensen, 1954). That haemoglobin acts catalytically under anaerobic conditions to split hydroxylamine into nitrogen, ammonia and water (Colter & Quastel, 1950) is a fact, however, which may minimize the antibacterial role of hydroxylamine in meats.

Another sphere in which the antibacterial activity of micrococci is of importance is the laboratory itself. Buttiaux (1958) has shown that it is impossible to isolate the most important bacterial species (a *Vibrio* sp.) found in French ham curing brines by using ordinary salt containing media because micrococci, with which it is associated, inhibit its growth. To isolate this organism it is necessary to add penicillin to the medium to inhibit the micrococci. This observation raises the question as to whether this is a common phenomenon; are estimates of the proportions of micrococci based on isolations from colonies on plates of medium carrying a mixed microflora generally too high for this reason?

4. THE USE OF CLASSIFICATIONS OTHER THAN THAT OF BERGEY'S *MANUAL*

Since the storage life of Wiltshire bacon is terminated by the development of a bacterial slime in which at least half the organisms are micrococci, it would be most useful to know, with a view to their eventual eradication, whether they originate on the sides of pork or are introduced in the pickling process.

By distributing 168 strains isolated from pork sides, bacon brines, immature bacon (i.e. straight out of tank) and mature bacon (slime) into the subgroups of Shaw *et al.* (1951) it has been shown (Kitchell, 1958) that affinities exist between the pork and bacon microfloras. Both contain more subgroup 2 and 5 strains than the brine. It is possible, therefore, that the spoilage types come from pork. However, the majority of isolates from all sources belong to subgroup 3, *Staph. lactis*. Thus, without a breakdown of subgroup 3, further progress towards answering this question will be difficult. Nevertheless, application of the same scheme to 193 strains of micrococci from vacuum packed bacon before and after storage to destruction at 5°, 20° and 30° (Kitchell, unpublished data) demonstrated the temperature dependent changes in the proportions of micrococci and coagulase negative staphylococci noted by Ingram (1960) and Cavett (1962). At 30°, 30% of the isolates fell into subgroup 2 (*Staph. saprophyticus*), whereas at 20° only 8% belonged to this subgroup. At the beginning of the experiment the freshly sliced bacon carried 9% of subgroup 2 strains.

Recognizing that most of his micrococci belonged to subgroup 3, Pohja (1960) proceeded to classify them further on the basis of salt tolerance. He found that strains of potential value as starter cultures in dry sausage manufacture fell into salt tolerant (15% NaCl at 20°) subgroups which were further separated on their optimum salt concentration for growth and acid production from arabinose. There have been no reports on the use of this scheme by other workers and thus the value to meat microbiologists of Pohja's subdivision of the *Staph. lactis* subgroup has yet to be confirmed.

5. THE FLUORESCENT ANTIBODY TECHNIQUE

A more direct method of locating the source of micrococci found in the slime on sides of bacon has been investigated (Kitchell, unpublished data) but without success; this was the fluorescent antibody technique of Coons & Kaplan (1950) and Chadwick, McEntegart & Nairn (1958) as applied by Hobson & Mann (1957). Failure was due largely to the difficulty of preparing antisera with high titres of antibodies against the 6 spoilage strains belonging to subgroup 3 which were injected into rabbits. The prepared antisera agglutinated strain NCTC 4351 (subgroup 3). This organism also agglutinated at all dilutions tested in controls set up with pre-inoculation sera. The homologous strains did not agglutinate with pre-inoculation sera. In tests with a few other NCTC strains representing subgroups 2, 3, 4 and 5 it was found that the reaction with both antisera and pre-inoculation sera was specific for subgroup 3; an interesting but unhelpful observation.

Further difficulties arose because the various manipulations involved in conjugating the antisera with fluorescent tracers reduced the already low titres. Given sufficiently high titres it should be possible, by various adsorption techniques, to derive satisfactory antisera. Facilities were not readily available to pursue this further, but the method has not been discounted as a possible way of locating strains of micrococci of particular interest.

6. CONCLUSIONS

The outstanding need in this field is more information about the nature and activities of micrococci, not only with a view to establishing a system of classification of value to those wishing to determine the distribution and source of different species, but also to provide basic information on the physiology of bacteria subject to a rather wide range of temperatures, NaCl concentrations and concentrations of other curing agents such as nitrate and nitrite. The indications that growth proceeds at concentrations of NaCl well above that at which proteolytic enzymes are inhibited draws attention to the need to know whether carbohydrate and lipid metabolism are similarly affected. Only when this knowledge is available will it be possible to determine the role of micrococci in cured meats and meat products: then and not now would be the appropriate time for the writing of this paper.

7. REFERENCES

Alm, F., Erichsen, I. & Molin, N. (1961). The effect of vacuum packaging on some sliced processed meat products as judged by organoleptic and bacteriological analysis. *Food Tech., Champaign*, **15**, 199.

Baird-Parker, A. C. (1962). The occurrence and enumeration, according to a new classification, of micrococci and staphylococci in bacon and on human and pig skin. *J. appl. Bact.* **25**, 352.

Baird-Parker, A. C., (1963). A classification of micrococci and staphylococci based on physiological and biochemical tests. *J. gen. Microbiol.*, **29**, (in press).

Breed, R. S., Murray, E. G. D. & Hitchens, A. P. (1948). Bergey's *Manual of Determinative Bacteriology*, 6th ed. London: Baillière, Tindall & Cox.

Brooks, J., Haines, R. B., Moran, T. & Pace, J. (1940). The function of nitrate, nitrite and bacteria in the curing of bacon and hams. *Spec. Rep. Fd Invest. Bd. Lond.*, no. 49.

Brooks, R. F. & Henrickson, R. L. (1956). Microflora of prepackaged luncheon meats. *Bull. Mo. agric. Exp. Sta.* no. 611.

Buttiaux, R. (1958). Technique simple d'examen bactériologique des saumures de jambon et ses résultats. *2nd Int. Symp. Fd Microbiol., Cambridge*, p. 137. London: H.M.S.O.

Cavett, J. J. (1962). The microbiology of vacuum packed sliced bacon. *J. appl. Bact.* **25**, 282.

Cesari, E.-P. (1919). La maturation du saucisson. *C.R. Acad. Sci., Paris*, **168**, 802.

Chadwick, C. S., McEntegart, M. G. & Nairn, R. C. (1958). Fluorescent protein tracers—a simple alternative to fluorescein. *Lancet* **i**, 412.

Colter, J. S. & Quastel, J. H. (1950). Catalytic decomposition of hydroxylamine by haemoglobin. *Arch. Biochem.* **27**, 368.

Coons, A. H. & Kaplan, M. H. (1950). The localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. *J. exp. Med.* **91**, 1.

Coretti, K. (1956). Veränderung des Keimgehaltes während der Reifung von Rohwurst. *Fleischwirtschaft* **8**, 197.

Deibel, R. H. & Niven, C. F. (Jr.) (1957). *Pediococcus cerevisiae*: a starter culture for summer sausage. *Bact. Proc.* p. 14.

Deibel, R. H., Niven, C. F. (Jr.) & Wilson, G. D. (1961). Microbiology of meat curing. III. Some microbiological and related technological aspects in the manufacture of fermented sausages. *Appl. Microbiol.* **9**, 156.

Dussault, H. P. (1958). The salt tolerance of bacteria from lightly salted fish. *2nd Int. Symp. Fd Microbiol., Cambridge*, p. 61. London: H.M.S.O.

EDDY, B. P. (1958). The effects of salt concentration and temperature on nitrite production and metabolism. *2nd Int. Symp. Fd Microbiol., Cambridge*, p. 87. London: H.M.S.O.

EDDY, B. P. (1961). The Voges-Proskauer reaction and its significance: a review. *J. appl. Bact.* **24**, 27.

EDDY, B. P., GATHERUM, D. P. & KITCHELL, A. G. (1960). Bacterial metabolism of nitrate and nitrite in maturing bacon. *J. Sci. Fd Agric.* **11**, 727.

EMPEY, W. A. & SCOTT, W. J. (1939). Investigations on chilled beef. Part I. Microbial contamination acquired in the meat works. *Bull. Counc. sci. industr. Res. Aust.* no. 126.

EVANS, J. B. & NIVEN, C. F. (Jr.) (1955). Slime and mould— problems with pre-packaged meat products. *Bull. Amer. meat Inst. Found.* no. 24.

EVANS, J. B., BRADFORD, W. L. (Jr.) & NIVEN, C. F. (Jr.) (1955). Comments concerning the taxonomy of the genera *Micrococcus* and *Staphylococcus*. *Int. Bull. bact. Nomen. Taxon.* **5**, 61.

GARRARD, E. H. & LOCHHEAD, A. G. (1939). A study of bacteria contaminating sides of Wiltshire bacon with special consideration of their behaviour in concentrated salt solutions. *Canad. J. Res.* **17** D, 45.

GREENWOOD, D. A., LEWIS, W. L., URBAIN, W. M. & JENSEN, L. B. (1940). The haem pigments of cured meats. IV. Role of sugars in colour of cured meats. *Food Res.* **5**, 625.

GUTHEIL, N. C. (1958). Micrococci of brine and dry cured beef (charque). *2nd Int. Symp. Fd Microbiol., Cambridge*, p. 183. London: H.M.S.O.

HAINES, R. B. (1933*a*). Observations on the bacterial flora of some slaughterhouses. *J. Hyg., Camb.*, **33**, 165.

HAINES, R. B. (1933*b*). The bacterial flora developing on stored lean meat, especially with regard to 'slimy' meat. *J. Hyg., Camb.*, **33**, 175.

HANKINS, O. G., SULZBACHER, W. L., KAUFFMAN, W. R. & MAYO, M. E. (1950). Factors affecting the keeping qualities of bacon. *Food Tech., Champaign*, **4**, 33.

HANSEN, N.-H. (1960). Quality deterioration and bacterial growth in prepacked bacon. *Danish Meat Res. Inst., Roskilde*, publication no. 28.

HOBSON, P. N. & MANN, S. O. (1957). Some studies on the identification of rumen bacteria with fluorescent antibodies. *J. gen. Microbiol.* **16**, 463.

HOROVITZ-VLASOVA, L. M. & LIVSHITZ, M. I. (1934). Experimental study of the so-called sliminess of salt pork. *Chem. Abstr.* **28**, 1416.

INGRAM, M. (1934). The microbiology of pork and bacon. The microflora of the slime of bacon. *Rep. Fd Invest. Bd, Lond.*, p. 77.

INGRAM, M. (1952). Internal bacterial taints ('bone taint' or 'souring') of cured pork legs. *J. Hyg., Camb.*, **50**, 165.

INGRAM, M. (1955). The heat resistance of a micrococcus. *Ann. Inst. Pasteur, Lille*, **7**, 146.

INGRAM, M. (1960). Bacterial multiplication in packed Wiltshire bacon. *J. appl. Bact.* **23**, 206.

INGRAM, M. & HOBBS, B. C. (1954). The bacteriology of 'pasteurized' canned hams. *J.R. sanit. Inst.* **74**, 1151.

JENSEN, L. B. (1954). *The Microbiology of Meats*. 3rd ed. Champaign, Illinois: The Garrard Press.

JENSEN, L. B. & PADDOCK, L. S. (1940). Sausage treatment with lactobacilli. U.S. Patent no. 2,225,783.

JEPSEN, A. (1947). Bacteriology of pork. Microbial contamination acquired in the meat works. *K. VetHøjsk. Aarsskr.*, p. 40.

KAMIYA, S. (1960). Decomposition of ascorbic acid. IX. Decomposing mechanism of L-ascorbic acid by bacteria. 4. Investigations on the decomposition products. *Chem. Abstr.* **54**, 7798.

KAMIYA, S., YAMAMOTO, E. & NAKABAYASHI, T. (1960). Decomposition of ascorbic acid. V. Separation and microbiological properties of ascorbic acid-decomposing bacteria. *Chem. Abstr.* **54**, 7797.

KELLER, H. (1954). Die bakterielle Aromatisierung der Rohwurst. *Fleischwirtschaft* **6**, 125.

KELLER, H. & MEYER, E. (1954). Bakterielle Aromatisierung der Rohwurst (2. Beitrag). *Fleischwirtschaft* **6**, 453.

KITCHELL, A. G. (1958). The micrococci of pork and bacon and of bacon-curing brines. *2nd Int. Symp. Fd Microbiol., Cambridge*, p. 191. London: H.M.S.O.

LEISTNER, L. (1958*a*). Bakterielle Vorgänge bei der Pökelung von Fleisch. I. Der Keimgehalt von Pökellaken. *Fleischwirtschaft* **10**, 74.

LEISTNER, L. (1958*b*). Bakterielle Vorgänge bei der Pökelung von Fleisch. II. Günstige Beeinflussung von Farbe, Aroma und Konservierung des Pöklefleisches durch Mikroorganismen. *Fleischwirtschaft* **10**, 226.

LEISTNER, L. (1958*c*). Bakterielle Vorgänge bei der Pökelung von Fleisch. III. Die Verderbnis von Pökellaken. *Fleischwirtschaft* **10**, 530.

LEISTNER, L. (1959). Die Keimarten und Keimzahlen von Pökellaken. *Fleischwirtschaft* **11**, 726.

LERCHE, M. (1956). Mikroben in Rohwurst. *Berl. Münch. tierärztl. Wschr.* **69**, 91.

MORAN, T. & SMITH, E. C. (1929). Post-mortem changes in animal tissues—the conditioning or ripening of beef. *Spec. Rep. Fd Invest. Bd, Lond.*, no. 36.

NIINIVAARA, F. P. (1955). Über den Einfluss von Bakterienreinkulturen auf die Reifung und Umrötung der Rohwurst. *Acta agral. fennica.* **84**, 1.

NIINIVAARA, F. P. (1958). Observations on the significance of a *Micrococcus* strain in meat processing. *2nd Int. Symp. Fd Microbiol., Cambridge*, p. 187. London: H.M.S.O.

NIINIVAARA, F. P. (1962). Some aspects about using bacterial pure cultures in the manufacture of fermented meat products. 22nd Annual meeting. Inst. Fd Technsts, Miami, paper no. 39.

NIINIVAARA, F. P. & POHJA, M. S. (1957*a*). Die in Rohwurst vorkommenden Bakterien und ihre Bedeutung beim Reifungsprozess. *Fleischwirtschaft* **9**, 264.

NIINIVAARA, F. P. & POHJA, M. S. (1957*b*). Erfahrungen bei der Herstellung von Rohwurst mit Bakterienreinkulturen. *Fleischwirtschaft* **9**, 789.

OGILVY, W. S. & AYRES, J. C. (1953). Post-mortem changes in stored meats. V. Effect of CO_2 on microbial growth on stored frankfurters and characteristics of some microorganisms isolated from them. *Food Res.* **18**, 121.

POHJA, M. S. (1960). Micrococci in fermented meat products. Classification and description of 171 different strains. *Acta agral. fennica.* **96**, 1.

POHJA, M. S. & NIINIVAARA, F. P. (1960). Die Bedeutung einige starke proteolytische, zur Gattung *Bacillus* gehörige Stämme, bei der Reifung der Rohwurst. *Fleischwirtschaft* **12**, 932.

POHJA, M. S. & NIINIVAARA, F. P. (1961). Untersuchungen uber das Vermögen einige *Micrococcus-* und *Bacillus-* Stämme, Gelatine, Kasein, und Fleischeiweiss abzubauen. *Fleischwirtschaft* **13**, 52.

POHJA, M. S., RIGLER, F. & NIINIVAARA, F. P. (1960). Ein Verfahren zum Nachweis der Proteolyse im Fleisch. *Fleischwirtschaft* **12**, 834.

POLITI, I., COLLA, C. & CORBERI, E. (1944). The processes of ripening and the preservation of sausages. III. Investigations on the rapid ripening of smoked sausages. *Chem. Abstr.* **38**, 5994.

PROCEEDINGS (1958). *2nd Int. Symp. Fd Microbiol., Cambridge.* London: H.M.S.O.

ROCKWELL, G. E. & EBERTZ, E. G. (1924). How salt preserves. *J. infect. Dis.* **35**, 573.

ROSENFELD, W. D. & ZOBELL, C. E. (1947). Antibiotic production by marine microorganisms. *J. Bact.* **54**, 393.

SAVAGE, W. G., HUNWICKE, R. F. & CALDER, R. B. (1922). The bacteriology of canned meat and fish. *Spec. Rep. Fd Invest. Bd, Lond.*, no. 11.

SHAW, C., STITT, J. F. & COWAN, S. T. (1951). Staphylococci and their classification. *J. gen. Microbiol.* **5**, 1010.

SKOVGAARD, N. (1959). Bacteriology of canned meats. *Rep. Int. vet. Congr., Madrid*, p. 799.

STEINKE, P. K. W. & FOSTER, E. M. (1951*a*). Microbial changes in refrigerated liver sausage. *Food Res.* **16**, 245.

STEINKE, P. K. W. & FOSTER, E. M. (1951*b*). Effect of different artificial casings on the microbial changes in refrigerated liver sausage. *Food Res.* **16**, 289.

STEINKE, P. K. W. & FOSTER, E. M. (1951*c*). Effect of temperature of storage on microbial changes in liver sausage and bologna. *Food Res.* **16**, 372.

SU, T. L. (1948). Micrococcin. An antibacterial substance formed by a strain of *Micrococcus*. *Brit. J. exp. Path.* **29**, 473.

TARR, H. L. A. (1941). The bacteriostatic action of nitrites. *Nature, Lond.*, **147**, 417.

ULRICH, J. A. & HALVORSON, H. O. (1951). Chemical and microbial studies on sliced canned bacon. *Advanc. Fd Res.* **3**, 291.

WHITE, W. H. (1941). Canadian Wiltshire bacon. XVII. Rancidity in pork fat after frozen storage and conversion to bacon. *Canad. J. Res.* **19** D, 96.

ZANZUCCHI, A. & DELINDATI, G. (1956). Study on some aroma-producing bacterial strains isolated from raw sausages during the ripening process. *Chem. Abstr.* **50**, 4421.

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Morrison, R. B. (1962). *J. appl. Bact.* **25** (3), 432–435.

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THE COAGULASE TEST IN THE IDENTIFICATION OF PATHOGENIC STAPHYLOCOCCI

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1. Introduction

There are two kinds of pathogenic staphylococci: those that cause pyogenic infections and those responsible for toxic food poisoning. The same organism may show both these apparently unrelated effects, but a strain of staphylococcus with high pyogenic virulence and pathogenicity may produce little or no enterotoxin. Whether the reverse is true I am less certain, but irrespective of the kind of pathogenicity concerned the best means available for deciding whether a Gram-positive micrococcus is the pathogen *Staphylococcus pyogenes* is the coagulase test. In other words, does the isolate produce an enzyme which clots citrated human, rabbit and other bloods? If it does it is judged to be a pathogen. If we cannot be certain enough to bring in a verdict of 'guilty' we can at least reach the old Scottish legal verdict of 'Not proven', which implies a heavy suspicion of guilt! This is the current view of the significance of the coagulase test and to see how this dogmatic standpoint has been reached we must go back 50 years.

The credit for the discovery of coagulase is usually given to Loeb (1903). In fact all he found was that goose plasma, which clots slowly and spontaneously, clotted more quickly in the presence of staphylococci and many other species of bacteria, the cells acting as foci for fibrin deposition. The real discoverer of coagulase was Much (1908), and the paper describing his findings is a classic of microbiology. In this publication every important aspect of the blood clotting enzyme coagulase was discovered, partly discovered or predicted. However, it took the usual 10 years or so for other bacteriologists to realize that the presence of this highly specific enzyme was a fairly certain indication that a staphylococcus was pathogenic.

Throughout the 1920s and 1930s many investigations, some covering thousands of strains, were made into the relationship between coagulase production and pathogenicity. If I had to summarize in a sentence the scope of this work and the conclusions to be drawn therefrom, I should say that all sorts of properties of the staphylococcus were found to be associated with pathogenicity—pigment production, mannitol fermentation, gelatin liquefaction, α-haemolysin production, phosphatase production and so on—but no other property was so clearly associated with pathogenicity as the ability to clot citrated human, rabbit and other blood plasma.

Here we run into a philosophical difficulty. A pathogenic staphylococcus has become an organism which produces coagulase and *vice versa*. This, if not an axiom, has become at least a doctrinal tenet: but what does it mean? If I were to be completely frank, which for your peace of mind I shall try to avoid being, I should admit that we do not know. It is extremely difficult, even in the case of pyogenic staphylococci, to test for pathogenicity; with enterotoxin producing staphylococci it is almost impossible. In fact, to ensure that a staphylococcus isolated from food suspected of having caused poisoning is the cause, one needs as experimental animals several men or women who are prepared to swallow broth cultures and be wretchedly ill. But such experimental animals are not readily available, and as an alternative coagulase production is relied on as an index of pathogenicity. To do this is not to be completely irrational. As I remarked earlier, examination of thousands of strains isolated from pyogenic infections and from food eaten prior to attacks of food poisoning has shown that if there is no absolute certainty that coagulase production and pathogenicity are synonymous we can at least bring in the peculiarly American verdict of 'Guilty by association'.

Consideration of what is known about coagulase, and in particular about its role in, as opposed to its association with, pathogenicity, shows that intensive work spread over 50 years has failed to provide a key to its functions. A good deal is known about its nature (Tager, 1948; Duthie & Lorenz, 1952), about its mode of action *in vitro* (Smith & Hale, 1944), about its antigenicity (Lominski, 1949; Rammelkamp *et al.*, 1950; Tager & Hales, 1948), and about its production or lack of production in defined media (Lominski *et al.*, 1950). But fragments only are known about its role in infection (Hale & Smith, 1945), about its ability to clot blood *in vivo* (Smith & Johnstone, 1958) and, lately, about its ability to act as a protective antigen in group III staphylococcal infections (Lominski *et al.* 1962). In the short time available it would be impossible to discuss adequately the vast body of work covered by these few references. Instead I should like to deal with two aspects only: the mode of action of coagulase and the detection of its production.

2. MODE OF ACTION OF COAGULASE

As I stated earlier, coagulase has been known to bacteriologists for many years and used as a diagnostic test without any great understanding of how it achieves its blood clotting effect. The bulk of the evidence has always pointed to its *not* being identical with any of the components of plasma involved in the normal clotting process. It acts in the presence of substances which interfere with normal clotting, such as citrate, oxalate and heparin. The first real light on its mode of action came with the publication of that now classical paper by Wilson Smith and his colleague (Smith & Hale, 1944) which showed that coagulase was produced by the staphylococcus in an inactive form and that it reacted with activator (present in human and other bloods, but absent from the bloods of a few species) to give active coagulase. Wilson Smith was unable to show whether activator or coagulase was the quantitative precursor of active coagulase, and some doubt still exists today. About 10 years ago Goudie and I spent a great deal of time trying to settle this issue and, although most of the evidence pointed to coagulase standing in the same relationship to active coagulase as trypsinogen to trypsin, we did not find our proof rigorous enough to warrant publication.

3. TESTING FOR COAGULASE PRODUCTION

Many variations have been published of the standard coagulase test, but they fall into two classes, the tube test and the slide test.

(a) *The tube test*

This test is simplicity itself. A large number of staphylococcal cells, either from growth on solid media or better in the form of broth culture, are added to a 1:5 saline dilution of human or rabbit plasma containing an anticlotting agent. The test tube is incubated at 37° and examined frequently in the first 3 hr for clotting and again after 18–24 hr. The snags are few and can be guarded against. The clot may form and subsequently be digested by staphylococcal fibrinolysin, so that frequent inspection is necessary. Some strains not only produce coagulase but also a coagulase destroying enzyme (Lominski, Morrison & Smith, 1955); this destruction is prevented by egg white trypsin inhibitor. If one is unwise enough to want a quick answer with a mixed culture of *Staph. pyogenes* and other organisms, the other species present may utilize the citrate and thereby allow the normal clotting process to occur (Harper & Conway, 1948); heparin instead of citrate will obviate this. Some group D streptococci appear to produce a coagulase in small amounts (Wood, 1959); one should, however, be able to tell an enterococcus from a staphylococcus. These, I think, are the main sources of confusion.

(b) *The slide test*

In this test (Cadness-Graves *et al.*, 1943) a heavy suspension of staphylococci is mixed with a drop of plasma or fibrinogen solution on a microscope slide, and if clumping occurs within a few seconds (less than 1 minute) then the strain is judged to be coagulase positive. This test avoids many of the difficulties met with in the tube test and for most purposes is adequate: it should not give false positives, but its use may entail missing about 10% of weakly coagulase positive strains.

4. CONCLUDING REMARKS

There is some doubt whether the same coagulase is responsible for positive reactions in the two tests, but time does not allow a discussion of the properties of free and bound coagulase. Nor does it permit me to deal with the many other aspects of coagulase which I find much more fascinating than the techniques of testing for its presence or absence, but perhaps some of these can be touched upon in the discussion of this paper.

5. REFERENCES

CADNESS-GRAVES, B., WILLIAMS, R., HARPER, G. J. & MILES, A. A. (1943). Slide-test for coagulase-positive staphylococci. *Lancet* **i**, 736.

DUTHIE, E. S. & LORENZ, L. (1952). Staphylococcal coagulase: mode of action and antigenicity. *J. gen. Microbiol.* **6**, 95.

HALE, J. H. & SMITH, W. (1945). The influence of coagulase on the phagocytosis of staphylococci. *Brit. J. exp. Path.* **26**, 209.

HARPER, E. M. & CONWAY, N. S. (1948). Clotting of human citrated plasma by Gram-negative organisms. *J. path. Bact.* **60**, 247.

LOEB, L. (1903). The influence of certain bacteria on the coagulation of the blood. *J. med. Res.* **10**, 407.

LOMINSKI, I. (1949). Susceptibility and resistance to staphylococcal infection. *J. gen. Microbiol.* **3**, ix.

LOMINSKI, I., MORRISON, R. B. & SMITH, D. D. (1955). Potentiation and destruction of R variant coagulase by products of S variants of *Staphylococcus aureus*. *J. gen. Microbiol.* **13**, 446.

LOMINSKI, I., O'HEA, A. J., GOUDIE, J. G. & PORTER, I. A. (1950). Production of staphylocoagulase in a chemically defined medium. *Nature, Lond.*, **166**, 309.

LOMINSKI, I., SMITH, D. D., SCOTT, A. C., ARBUTHNOTT, J. P., GRAY, S., MUIR, D., TURNER, G. H. & HEDGES, C. K. (1962). Immunisation against experimental staphylococcal infection with coagulase-rich preparations. *Lancet* **i**, 1315.

MUCH, H. (1908). Über eine vorstufe der Fibrinfermentes in Kulturen von *Staphylokokkus aureus*. *Biochem.Z.* **14**, 143.

RAMMELKAMP, C. H. (Jr.), BADGER, G. F., DINGLE, J. H., FELLER, A. E. & HODGES, R. G. (1950). Antigenicity of cell-free coagulase. *J. infect. Dis.* **86**, 159.

SMITH, D. D. & JOHNSTONE, J. M. (1958). Coagulase activity *in vivo*. *Brit. J. exp. Path.* **39**, 165.

SMITH, W. & HALE, J. H. (1944). The nature and mode of action of staphylococcus coagulase. *Brit. J. exp. Path.* **25**, 101.

TAGER, M. (1948). Concentration, partial purification, properties and nature of staphylocoagulase. *Yale J. Biol. Med.* **20**, 487.

TAGER, M. & HALES, H. B. (1948). The experimental production of antibodies to staphylocoagulase. *J. Immunol.* **60**, 475.

WOOD, M. (1959). The clotting of rabbit plasma by group D streptococci. *J. gen. Microbiol.* **21**, 385.

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Alder, V. G., Gillespie, W. A. & Waller, L. J. (1962). *J. appl. Bact.* **25** (3), 436–440.

(Symposium on Staphylococci and Micrococci: Paper XII)

THE USE OF TELLURITE-EGG YOLK MEDIUM FOR THE ISOLATION AND IDENTIFICATION OF STAPHYLOCOCCI IN HOSPITALS

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1. Introduction

The object of this paper is to describe the use of tellurite-egg yolk agar (TEA) in clinical bacteriology. This medium has been of considerable value for isolating and identifying *Staphylococcus aureus* from infective material such as pus, sputum and urine and, in settle plates, for assessing aerial contamination by staphylococci in surgical wards.

The tellurite-egg yolk medium first described by Innes (1960) for the selective isolation of staphylococci from foodstuffs was slightly changed for clinical use (see below). Innes modified Ludlam's (1949) tellurite medium by reducing the content of Lemco and halving the concentration of tellurite. Since he had observed that all the enterotoxic strains of *Staph. aureus* studied by him gave a positive reaction in the egg yolk test of Gillespie & Alder (1952), and because most coagulase positive strains of *Staph. aureus* from human sources are egg yolk positive (Alder, Gillespie & Herdan, 1953), egg yolk was added to provide a differential culture medium for staphylococci of human origin. Sodium chloride (1%) was added to reduce the opacity of the egg yolk. Innes further observed that the growth of staphylococci on the tellurite medium was improved by the addition of egg yolk, which appeared to neutralize the inhibiting action of tellurite on staphylococci without reducing its inhibition of other bacteria.

2. MATERIALS AND METHODS

Tellurite-egg yolk agar (*TEA*). For simplicity, the basal medium was made according to Ludlam's original formula (instead of the modification introduced by Innes) as follows: Lab-Lemco (Oxoid), 10 g; peptone (Difco), 10 g; dipotassium hydrogen phosphate, 5 g; lithium chloride, 5 g; mannitol, 10 g; agar (Davis), 15 g; distilled water, 900 ml; pH 9·2. For use, the basal medium was melted and cooled to 50°; then the following reagents were added to each 900 ml quantity: sodium chloride, 10 g; sterile 1:400 aqueous potassium tellurite solution, 10 ml; concentrated egg yolk (Oxoid), 100 ml.

Clinical material. Specimens of pus, sputum, urine, etc., sent for routine bacteriological examination, were plated on TEA medium, in parallel with the usual plating on blood agar and MacConkey's agar.

Aerial contamination by staphylococci was investigated by exposing 3 in. plates of nutrient agar and tellurite-egg yolk agar side by side in surgical wards for 3 hr. This was done twice a week for a period of 5 months.

3. RESULTS

(a) *The growth and appearance of* Staph. aureus *isolated from clinical specimens plated on tellurite-egg yolk medium*

Numerous strains of *Staph. aureus* were isolated from routine clinical specimens subcultured on TEA medium. Without exception all grew well. The colonial appearances were nearly always quite distinct from any other organisms which succeeded in growing on the medium. Colonies of *Staph. aureus*, after incubation for 24 hr at 37°, were smooth, grey-black in colour with a slightly paler, even margin; they measured about 2 mm in diameter. Opacity developed in the medium around and underneath the colonies of most strains. Strains which gave a positive egg yolk reaction by the broth method (Gillespie & Alder, 1952) produced marked opacity on the plates, but some strains which were egg yolk negative in broth also produced some opacity on the plates, though this usually was less pronounced.

In contrast to the invariably good growth of coagulase positive staphylococci, most strains of *Staph. albus* (coagulase negative) and micrococci grew poorly, with colonies which were blacker and smaller than those of *Staph. aureus* and easily distinguishable from them. However, a few coagulase negative strains did grow well on TEA plates, but without producing opacity in the medium or grey margins around the colonies. In practice it was nearly always easy to distinguish between colonies of *Staph. aureus* and *Staph. albus*, but their identity was always confirmed by performing the coagulase test after subculture on nutrient agar. The slide coagulase test was unreliable when performed directly on colonies from TEA plates.

Most of the species of bacteria encountered in clinical specimens did not grow at all on TEA, but some coli-aerogenes bacteria and *Pseudomonas pyocyanea* and *Proteus* spp. grew feebly, forming minute colonies which were visible after incubation for 2 days and were readily distinguishable from *Staph. aureus*. Colonies of *Proteus* spp. did not swarm.

The practical value of the medium was brought out by comparing the results obtained when pus from infected wounds and abscesses, sputum and infected urine were cultured on TEA and on blood agar plates (Table 1). Most of the specimens contained mixtures of organisms, in which staphylococci were sometimes greatly outnumbered. Of the 180 specimens cultured, 108 yielded *Staph. aureus* on TEA compared with 95 on blood agar. The failure of blood agar to recover *Staph. aureus* from 13 specimens was due to the presence of numerous Gram-negative rods which crowded out the staphylococci or swarmed over them. The selective quality of TEA was often strikingly demonstrated, even when the specimen contained very few staphylococci and many other organisms.

Table 1. *The results of culturing* 180 *clinical specimens on blood agar and on TEA plates*

Source of organisms	No. of specimens	Nos. of organisms isolated on							
		Blood agar				Tellurite-egg yolk agar (TEA)			
		Staph. aureus	Micrococci and *Staph. albus*	Gram-negative rods	Streptococci, yeasts, etc.	*Staph. aureus*	Micrococci and *Staph. albus*	Gram-negative rods	Streptococci, yeasts, etc.
Wounds and urine	89	32	17	35	16	38	2	1	1
Sputum	24	7	0	10	14	14	1	2	0
Abscesses	67	56	3	2	4	56	0	1	0
Totals	180	95	20	47	34	108	3	4	1

The improved growth of *Staph. aureus* resulting from the addition of egg yolk to Ludlam's medium, as observed by Innes, was confirmed by growing several strains of staphylococci on both Ludlam's medium and TEA containing the same concentrations of potassium tellurite (0·005 g/100 ml). The results were similar when the tellurite concentration was reduced to 0·0025 g/100 ml (Plate 1). Growth on TEA did not alter the phage patterns of the staphylococci.

(b) *Direct antibiotic sensitivity tests on tellurite-egg yolk agar plates*

Direct sensitivity tests by the agar diffusion disc technique can be carried out on TEA plates even when other organisms are present in the specimen, and may be of value in obtaining provisional results in urgent cases. Plate 2 shows the result of a direct sensitivity test on TEA and on blood agar, with a wound swab which contained *Proteus* as well as *Staph. aureus*. The swarming *Proteus* obscured the result on blood agar but not on the TEA.

The limitations of disc sensitivity tests, due to the variation in size of inhibition zones with size of inoculum and with the lag period and generation time of the organisms (Linton, 1961), apply equally to tests done on TEA plates as to tests done on other media. Because of the slower growth of staphylococci on TEA than on blood agar, zones of inhibition are generally larger. A control plate of TEA, inoculated with a culture of known sensitivity, must always be incubated for comparison.

(c) *The use of tellurite-egg yolk settle plates in epidemiological studies*

Settle plates of nutrient agar and tellurite-egg yolk agar were exposed together in several wards, and the colonies of *Staph. aureus* developing after incubation at 37° for 40–45 hr were counted. The counts on TEA plates averaged 5·1 colonies/plate, compared with an average of 2·9 colonies on nutrient agar (Table 2). The higher counts on TEA may have been partly due to easier identification of the colonies as well as to the suppression of other organisms. The identity of the colonies of presumptive *Staph. aureus* on TEA settle plates was confirmed by picking off a proportion of them for antibiotic sensitivity tests and coagulase production. Not less than 25% of all colonies present were checked in this way.

Table 2. *The counts of* Staph. aureus *obtained on nutrient agar and TEA settle plates exposed simultaneously in surgical wards*

Medium	No. of plates exposed	*Staph. aureus* colony count	
		Total	Average/plate
Nutrient agar	147	428	2·9
TEA	141	725	5·1

Egg yolk positive staphylococci could usually be identified by the large amount of opacity around the colonies on TEA plates. However, some egg yolk negative staphylococci produced slight opacity, and in practice it was not easy to distinguish with certainty between egg yolk positive and negative strains. TEA plates were therefore less satisfactory than egg yolk glucose broth for determining the egg yolk reaction of staphylococci for epidemiological purposes (Alder, Gillespie & Thompson, 1955).

4. Discussion

The main disadvantage, or rather limitation, of TEA in a clinical laboratory is that it is highly selective for *Staph. aureus*. It will therefore detect small numbers of staphylococci in a specimen such as sputum, and everything else will be suppressed. The significance of the staphylococci present might therefore be exaggerated if the medium were used alone. Hence it should be used as an adjunct to other media and not replace them. Used in this way it has been of great value in detecting coagulase positive staphylococci in complicated mixed infections.

Another disadvantage of the medium is that the slide coagulase test cannot be performed on colonies taken directly from a plate. However, the distinctive colonial appearance of *Staph. aureus* is easy to recognize and its identity can then be readily confirmed by microscopy and subculture.

Growth of *Staph. aureus* on TEA plates is slower than on blood agar, and incubation for a full 24 hr period is usually necessary to allow the typical appearance of the colonies to develop. However, small grey colonies can usually be seen after overnight incubation for 15–18 hr; if some are seen, they are almost certain to be *Staph.*

aureus, since no other common organism can grow as quickly on the medium. Hence, despite the comparatively slow growth of staphylococci on TEA, its selective quality sometimes allowed staphylococci in mixed infections to be identified earlier than on blood agar plates.

For the enumeration of air borne staphylococci, TEA plates were more reliable than nutrient agar plates.

5. SUMMARY

The use of tellurite-egg yolk agar (TEA) in clinical bacteriology has been evaluated. It was found to be valuable as a selective medium for isolating *Staph. aureus* from specimens containing other organisms, and especially when the staphylococci were outnumbered by coli-aerogenes bacteria, *Proteus* spp. or *Ps. pyocyanea*.

The medium was also of value in epidemiological studies of hospital cross infection, for the isolation of staphylococci from carrier sites and for counting air borne staphylococci on settle plates.

6. ACKNOWLEDGMENT

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7. REFERENCES

ALDER, V. G., GILLESPIE, W. A. & HERDAN, G. (1953). Production of opacity in egg-yolk broth by staphylococci from various sources. *J. Path. Bact.* **66**, 205.

ALDER, V. G., GILLESPIE, W. A. & THOMPSON, M. E. M. (1955). Virulence and phage patterns of antibiotic-resistant staphylococci in a hospital. *J. Path. Bact.* **70**, 503.

FAIRBROTHER, R. W. & MARTYN, G. (1951). The disc technique for determining sensitivity to the antibiotics. *J. clin. Path.* **4**, 374.

GILLESPIE, W. A. & ALDER, V. G. (1952). Production of opacity in egg-yolk media by coagulase positive staphylococci. *J. Path. Bact.* **64**, 187.

INNES, A. G. (1960). Tellurite-egg agar, a selective and differential medium for the isolation of coagulase positive staphylococci. *J. appl. Bact.* **23**, 108.

LINTON, A. H. (1961). Interpreting antibiotic sensitivity tests. *J. med. lab. Techn* **18**, 1.

LUDLAM, G. B. (1949). A selective medium for the isolation of *Staphylococcus aureus* from heavily contaminated material. *Mon. Bull. Ministr. Hlth Lab. Serv.* **8**, 15.

EXPLANATION OF PLATES

PLATE 1. Cultures (24 hr) of *Staph. aureus*: (*a*) and (*c*), on Ludlam's medium; (*b*) and (*d*), on tellurite-egg yolk agar (TEA).

(*a*) and (*b*) on media containing 0·0025 g of potassium tellurite/100 ml.

(*c*) and (*d*) on media containing 0·005 g of potassium tellurite/100 ml.

The TEA was made by adding egg yolk and sodium chloride to Ludlam's medium.

PLATE 2. Sensitivity tests performed by an agar diffusion technique based on the method of Fairbrother & Martyn (1951) on a mixed culture of *Staph. aureus* and a *Proteus* sp.

(*a*) Sensitivity tests on blood agar.

(*b*) Sensitivity tests on TEA.

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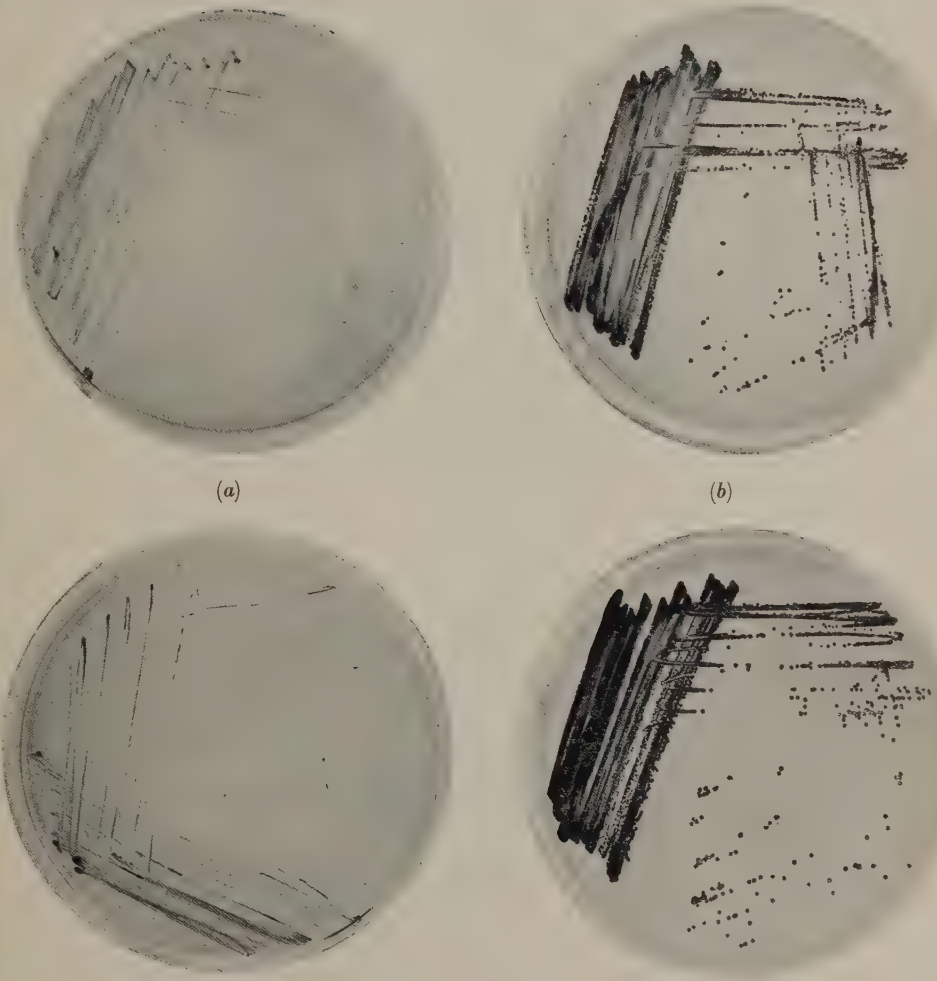
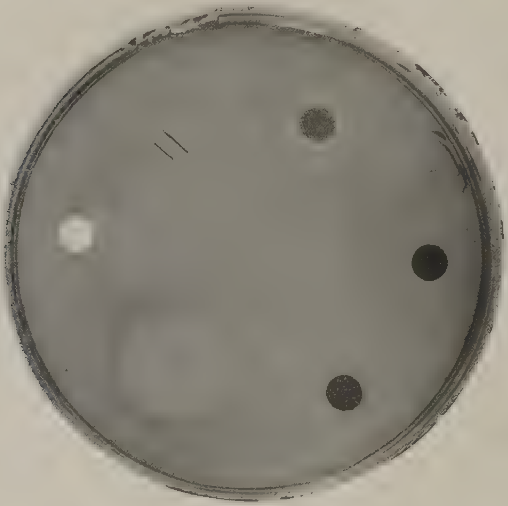


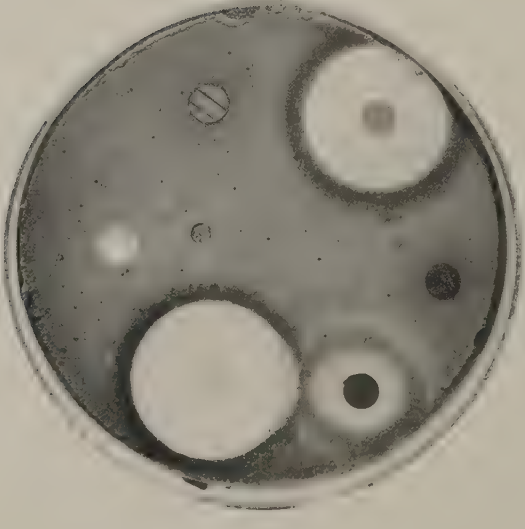
(a) (b)

(c) (d)

V. G. Alder, W. A. Gillespie & L. J. Waller — The Use of Tellurite-Egg Yolk Medium for the Isolation and Identification of Staphylococci in Hospitals. Plate 1



(*a*)



(*b*)

V. G. ALDER, W. A. GILLESPIE & L. J. WALLER — THE USE OF TELLURITE-EGG YOLK MEDIUM FOR THE ISOLATION AND IDENTIFICATION OF STAPHYLOCOCCI IN HOSPITALS. PLATE 2

Baird-Parker, A. C. (1962). *J. appl. Bact.* **25**, (3), 441–444.

(Symposium on Staphylococci and Micrococci: Paper XIII)

THE PERFORMANCE OF AN EGG YOLK-TELLURITE MEDIUM IN PRACTICAL USE

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SUMMARY: Observations by seven laboratories that have used egg-tellurite-glycine-pyruvate agar (ETGPA) (Baird-Parker, 1962) for isolating *Staph. aureus* from foods are reported and discussed. The results indicate that ETGPA is superior to media currently used for detecting this organism. The main disadvantages of the medium are the similar appearance of *Staph. aureus* shown by some strains of *Proteus vulgaris* and the occasional finding of egg yolk nonclearing strains of *Staph. aureus*.

Since the publication of the description of egg-tellurite-glycine-pyruvate agar (ETGPA) (Baird-Parker, 1962) several laboratories have made use of the medium for detecting *Staphylococcus aureus* in food. Seven of these have kindly submitted their results with comments on its use in food investigations, and this paper is based on the results received. Table 1 gives the products that have been tested, the methods of testing and comments as to the efficiency of the medium.

The general opinion is that ETGPA is very useful for detecting small numbers of *Staph. aureus* in foods and is superior in this respect to media currently used for this purpose. Most workers have reported excellent correlation between coagulase production and ability of *Staph. aureus* to grow on the medium as a black colony surrounded by a clear zone. Some workers, however, have found this correlation to be not as high as expected. Thus Stewart & Patterson (1963) report that 8·5% of *Staph. aureus* isolates on ETGPA did not clear egg yolk and also that 70% of colonies with the appearance of *Staph. aureus* on ETGPA were in fact coagulase negative. Probably the main reason why these workers gave a poor account of the performance of the medium was that *Proteus vulgaris* strains which showed similar growth to *Staph. aureus* on ETGPA were present in their samples. The present author has examined 3 strains of *Pr. vulgaris* supplied by Mr. J. Patterson and agrees that their appearance on ETGPA can be confused with that of *Staph. aureus* in the absence of microscopical observation. Attempts were made to obtain macroscopical differentiation of *Pr. vulgaris* on ETGPA. Partial success was obtained by including 0·1% of phenylalanine in the medium and detecting *Pr. vulgaris* by its ability to utilize this compound with the release of phenylpyruvic acid (Hormaeche & Peluffo, 1959). However, growth of *Pr. vulgaris* on ETGPA was not always sufficient for the detection of phenylpyruvic acid production by an *in situ* colony. Streaking a suspected colony of this organism on to the phenylalanine medium cited by

Hormaeche & Peluffo (1959) gave a positive result after incubation for 6 hr at 37°; *Staph. aureus* was always negative. With regard to the growth of *Proteus* spp. on selective media for isolating *Staph. aureus* it should be noted that Deneke & Blobel (1962) found that this organism grew on the tellurite-glycine agar of Zebovitz, Evans & Niven (1955) and on the polymyxin agar of Finegold & Sweeny (1961). It has also been pointed out that the egg yolk-azide agar of Hopton (1961) suffers in this respect.

Table 1. *Results reported by laboratories using ETGPA for isolating* Staphylococcus aureus *from foods*

Product	Method of testing	Comments
Frozen meat and fish products	Volumes of 0·1 or 1·0 ml of macerate spread on ETGPA	Most workers find method extremely satisfactory; except Stewart & Patterson (1962) (see text).
Freeze dried products	As above	Good recovery, 100% correlation between coagulase and appearance on ETGPA; recovery of very small numbers of *Staph. aureus* better than on mannitol-salt agar.
Meat pies and other cooked products	Samples enriched by incubating overnight at 37° in Robertson's meat broth + 10% of NaCl (Maitland & Martyn, 1948); diluted 1:10 and 1 ml spread on ETGPA: alternatively, 0·1 ml of macerate spread directly on to ETGPA	Satisfactorily used on a large number of foods; superior to mannitol-salt agar and phenolphthalein diphosphate agar.
Sausage and bacon	Volumes of 1·0 ml of macerate spread on ETGPA	Good; far better recovery than using previously published methods.
Curing brine	Volumes of 0·1 ml of a 1:10 dilution of curing brine spread on ETGPA	All colonies showing typical appearance were coagulase positive, with one exception (see text).
Fish	Volumes of 0·1 ml of macerate spread on ETGPA	Good recovery but no comparison made with other methods.
Meat, fish, cheese, egg, etc. sandwiches	Volumes of 0·1 or 1·0 ml of macerate spread on ETGPA	Good recovery of *Staph. aureus*; possible to detect these organisms on ETGPA when recovery impossible on blood agar.
Milk and products containing milk	Volumes of 0·1 ml of a dilution spread on ETGPA	Appears to be satisfactory; see Sharpe, Neave & Reiter (1962).

Most laboratories have reported good recovery of *Staph. aureus* on ETGPA. One laboratory reported that ETGPA allowed the growth of very small numbers of *Staph. aureus* definitely better than mannitol-salt agar. Most found that although the lag period was sometimes longer on ETGPA than on a nonselective medium, counts were similar after incubation at 37° for 24–48 hr. However, Stewart & Patterson (1963) claim that the three strains of *Staph. aureus* they tested showed poor recovery on the medium even after incubation for 48 hr. The present author has compared the growth of these strains on ETGPA, peptone-yeast extract-glucose agar and blood agar. Counts on ETGPA were similar to or better than those on the glucose agar, although generally slightly lower than those on blood agar.

Poor growth on ETGPA may be caused by a number of factors. In preparing the medium care must be taken to adjust the pH to 7·2 before autoclaving and to avoid wide fluctuations in pH during adjustment. Failure to do this will result in a precipitate developing; such media may be inhibitory. The tellurite concentration is also critical and batch variation may occur. The amount of tellurite can be standardized using the method suggested by Walper, Tucker & Appleman (1962). The sodium pyruvate solution should not be stored for longer than 1 month at 5°. Plates should be used within 24 hr of pouring. One worker has reported that inadequately washed Petri dishes may cause inhibition.

Some batches of ETGPA have failed to demonstrate the typical clearing of *Staph. aureus*. The reason for this has been traced to variation between batches of Oxoid egg yolk emulsion. Oxoid Ltd. are aware of this problem and all batches of egg yolk are now tested for clearing by *Staph. aureus*. Some workers have found strains of *Staph. aureus* which on primary isolation failed to clear egg yolk, even after incubation for 48 hr on ETGPA prepared with a batch of egg yolk known to demonstrate clearing. One such strain submitted, when replated on ETGPA, gave rise to some colonies showing well defined clearing after 24 hr. This phenomenon is by no means uncommon and Chapman, Berens, Nilson & Curcio (1938) observed that an apparently nonhaemolytic culture of *Staph. aureus* may give rise to some haemolytic colonies when plated out.

Most strains of *Staph. aureus* show typical clearing on ETGPA after incubation at 37° for 24–30 hr. Positive colonies should be noted at this time and then plates reincubated for a further 18–24 hr to detect late egg yolk clearers. It is recommended that late egg yolk clearing colonies should be tested for coagulase production as, after incubation for 48 hr on ETGPA, egg yolk clearing strains of *Pr. vulgaris* and some coagulase negative cocci may show a similar appearance to *Staph. aureus*.

In detecting coagulase it may be an advantage to use both the slide and tube tests. One strain submitted as coagulase negative but showing the typical appearance of *Staph. aureus* after incubation for 24 hr on ETGPA produced only bound coagulase. This anomalous strain was isolated from a curing brine.

In conclusion, ETGPA has been established to be a reliable method for isolating *Staph. aureus* from foods. The main drawbacks in the use of this medium are the similar appearance of *Pr. vulgaris* and *Staph. aureus* on the medium and the occasional occurrence of egg yolk nonclearing strains of *Staph. aureus*.

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REFERENCES

BAIRD-PARKER, A. C. (1962). An improved diagnostic and selective medium for isolating coagulase positive staphylococci. *J. appl. Bact.* **25**, 12.

CHAPMAN, G. H., BERENS, C., NILSON, E. L. & CURCIO, L. G. (1938). The differentiation of pathogenic staphylococci from non-pathogenic types. *J. Bact.* **35**, 311.

DENEKE, A. & BLOBEL, H. (1962). Fibrinogen media for studies on staphylococci. *J. Bact.* **63**, 533.

FINEGOLD, S. M. & SWEENY, E. E. (1961). New selective and differential medium for coagulase positive staphylococci allowing rapid growth and strain differentiation. *J. Bact.* **81**, 636.

HORMAECHE, E. & PELUFFO, C. A. (1959). Laboratory diagnosis of *Shigella* and *Salmonella* infections. *Bull. World Hlth Org.* **21**, 247.

HOPTON, J. (1961). A selective medium for the isolation and enumeration of coagulase positive staphylococci from foods. *J. appl. Bact.* **24**, 121.

MAITLAND, H. B. & MARTYN, G. (1948). A selective medium for isolating staphylococci based on the differentially inhibiting effect of increased concentrations of sodium chloride. *J. Path. Bact.* **60**, 553.

SHARPE, M. E., NEAVE, F. K. & REITER, B. (1962). Staphylococci and micrococci associated with dairying. *J. appl. Bact.* **25**, 403.

STEWART, D. J. & PATTERSON, J. T. (1963). The isolation of coagulase positive *Staphylococcus aureus* certain from meat products. *Int. Congr. Fd Sci. Technol., London*. New York: Gordon & Breach.

WALPER, J. F., TUCKER, F. L. & APPLEMAN, M. D. (1962). Use of thioacetamide for the quantitatitive determination of tellurite in biological media. *Analyt. Biochem.* **3**, 298.

ZEBOVITZ, E., EVANS, J. B. & NIVEN, C. F. (Jr.) (1955). Tellurite-glycine agar; a selective plating medium for the quantitative detection of coagulase-positive staphylococci. *J. Bact.* **70**, 686.

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STEEL, K. J. (1962). *J. appl. Bact.* **25** (3), 445–447.

THE OXIDASE ACTIVITY OF STAPHYLOCOCCI

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SUMMARY: By the use of Kovacs' (1956) test, oxidase activity was demonstrated in 23 of 66 strains of coagulase negative staphylococci (or micrococci) but in none of 82 strains of coagulase positive staphylococci. Less sensitive methods showed fewer reactions or failed to demonstrate them at all. Oxidase activity could not be correlated with other biochemical features.

THE ABSENCE of oxidase activity in staphylococci was reported by Steel (1961). Of 97 strains tested by Kovacs' (1956) method, only one (a strain of *Staphylococcus lactis*) showed any oxidase activity. Steel (1961) recognized three reactions: first, a positive reaction in which the blue colour developed within 10 sec; second, a delayed positive reaction in which the colouration developed in 10–60 sec; and third, a negative reaction in which colouration took longer than 60 sec or failed to develop at all.

The late Dr. B. P. Eddy of the Low Temperature Research Station, Cambridge (pers. comm.) also recognized three reactions: first, a positive reaction as above; second, a slow positive reaction in which the reagent is slowly oxidized, the blue colour taking 30–120 sec to reach its maximum intensity; and third, a negative reaction in which not only is the reagent not oxidized by the bacteria but its autoxidation is inhibited and the reagent spot dries with a white patch in the position of the bacterial mass. He found the negative reaction to be characteristic of *Staph. aureus* and *Staph. saprophyticus* and the slow positive to occur with some strains of *Staph. afermentans*, *Staph. lactis* and *Staph. roseus*.

The strains tested by Steel (1961) were mostly coagulase positive (*Staph. aureus*) and Kovacs' test has now been applied to all coagulase negative strains in the National Collection of Type Cultures. The classification used is that of Shaw, Stitt & Cowan (1951) and the results are shown in Table 1.

Eddy (pers. comm.) found that the addition of 0·1% of ascorbic acid to the reagent used in Kovacs' test permits easier distinction to be made between true positive reactions due to the presence of oxidase activity and slow reactions which may be due to autoxidation of the reagent.

Strains in which oxidase activity was demonstrated by Kovacs' (1956) method were examined by: a modification of Kovacs' method in which the reagent contained 0·1% of ascorbic acid; Gaby & Hadley's (1957) 'cytochrome oxidase' test using broth cultures; and Ewing & Johnson's (1960) method using agar slope cultures. The results are shown in Table 2. Eddy's method is not more sensitive than Kovacs' original one but it makes the reactions easier to read and serves to exclude 'false positives'.

Table 1. *The oxidase activities of strains of staphylococci as revealed by Kovacs' (1956) method*

Species	No. of strains tested	Reaction*		
		+	(+)	−
Staph. aureus	82	0	0	82
Staph. saprophyticus	19	0	1	18
Staph. lactis	22	0	6	16
Staph. afermentans	16	0	12 (4 weak)	4
Staph. roseus	9	0	4 (2 weak)	5
Totals	148	0	23	125

* Reactions: +, positive within 10 sec; (+), positive within 10–60 sec; —, negative.

Table 2. *The oxidase activities of strains of staphylococci producing delayed positive reactions by Kovacs'* (1956) *method*

Species	No. of strains tested	Nos. of cultures positive by		
		Eddy's modification of Kovacs' method*	Gaby & Hadley's (1957) method†	Ewing & Johnson's (1960) method†
Staph. saprophyticus	1	1 (weak)	0	0
Staph. lactis	6	6	0	0
Staph. afermentans	12	10 (3 weak)	0	3 (2 weak)
Staph. roseus	4	4 (weak)	0	0
Totals	23	21	0	3

* Reaction positive if occurring within 10–60 sec. † Reaction positive if occurring in 2 min.

The absence of positive reactions with Gaby & Hadley's (1957) method and the smaller number with Ewing & Johnson's (1960) method probably reflects the decreased sensitivity of these methods which use the dimethyl *p*-phenylenediamine reagent, whereas Kovacs' method uses the more sensitive tetramethyl compound. Gaby & Free (1958), however, considered that Kovacs' method was over sensitive.

The true negative reactions of *Staph. aureus*, in which the drop of reagent dried on the filter paper without colouration, were thought to be a reflection of the reducing activity of this species. Broth cultures of all strains of *Staph. aureus* and *Staph. saprophyticus* tested were able to reduce both oxidized Kovacs' reagent and methylene blue. Strains of other species gave variable results in these two tests.

It was not possible to show any correlation of oxidase activity with these features, or with nitrate reduction, inhibition by, or reduction of, triphenyltetrazolium chloride (see Kennedy & Barbaro, 1952; Burns & Holtman, 1960; Jeffries, 1961), growth in the presence of KCN (method of Rogers & Taylor, 1961) or arginine, lysine and ornithine decarboxylase activity (Møller, 1955). Hugh & Leifson's (1953) medium for glucose metabolism was not satisfactory for all strains of staphylococci used (see Baird-Parker, 1962), but strains showing oxidase activity generally appeared to be oxidizers rather than fermenters.

It is concluded that it is possible to demonstrate oxidase activity in some strains of staphylococci (or micrococci according to Baird-Parker, 1962) if a sufficiently sensitive method is used. The possible taxonomic implications have not been considered.

REFERENCES

BAIRD-PARKER, A. C. (1962). The occurrence and enumeration, according to a new classification, of micrococci and staphylococci in bacon and on human and pig skin. *J. appl. Bact.* **25**, 352.

BURNS, J. & HOLTMAN, D. F. (1960). Biochemical properties of virulent and avirulent staphylococci. *Ann. N.Y. Acad. Sci.* **88**, 1115.

EWING, W. H. & JOHNSON, J. G. (1960). The differentiation of *Aeromonas* and C 27 cultures from Enterobacteriaceae. *Int. Bull. bact. Nomen. Taxon.* **10**, 223.

GABY, W. L. & FREE, E. (1958). Differential diagnosis of Pseudomonas-like microorganisms in the clinical laboratory. *J. Bact.* **76**, 442.

GABY, W. L. & HADLEY, C. (1957). Practical laboratory test for the identification of *Pseudomonas aeruginosa*. *J. Bact.* **74**, 356.

HUGH, R. & LEIFSON, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J. Bact.* **66**, 24.

JEFFRIES, C. D. (1961). Comparison of six physiologic characteristics of staphylococci from laboratory specimens. *Amer. J. clin. Path.* **36**, 114.

KENNEDY, E. R. & BARBARO, J. F. (1952). The inhibitory effect of triphenyltetrazolium on some strains of micrococci. *J. Bact.* **63**, 297.

KOVACS, N. (1956). Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature, Lond.*, **178**, 703.

MØLLER, V. (1955). Simplified tests for some amino acid decarboxylases and for the arginine dihydrolase system. *Acta path. microbiol. scand.* **36**, 158.

ROGERS, K. B. & TAYLOR, J. (1961). Laboratory diagnosis of gastro-enteritis due to *Escherichia coli*. *Bull. World Hlth Org.* **24**, 59.

SHAW, C., STITT, J. M. & COWAN, S. T. (1951). Staphylococci and their classification. *J. gen. Microbiol.* **5**, 1010.

STEEL, K. J. (1961). The oxidase reaction as a taxonomic tool. *J. gen. Microbiol.* **25**, 297.

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PIKE, E. B. (1962). *J. appl. Bact.* **25** (3), 448–455.

THE CLASSIFICATION OF STAPHYLOCOCCI AND MICROCOCCI FROM THE HUMAN MOUTH

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1. INTRODUCTION

MEMBERS of the family Micrococcaceae are of almost universal occurrence in the human mouth and are present in the saliva, the 'white matter' collecting upon tooth surfaces, the necrotic areas of the advancing carious lesion and in periapical abscesses. The classical literature on oral microbiology (Vignal, 1886; Miller, 1890; Goadby, 1903; Kligler, 1915) suggests that the commonest forms in the human mouth are golden and white pigmented staphylococci: *Gaffkya tetragena* and *Sarcina lutea* are regular members of the flora, and chromogenic micrococci are found occasionally. No recent taxonomic study of the oral Micrococcaceae appears to have been made and this may be the cause of the inaccurate nomenclature, common in dental literature, which impedes comparison of different authors' findings. Morris (1954) and Taplin & Goldsworthy (1958) found existing taxonomic schemes inadequate for classifying these oral cocci, since many atypical strains were encountered.

This paper presents the results of a taxonomic comparison of oral Micrococcaceae with those isolated from other sources. It has been the object of this study to devise a straightforward diagnostic scheme for these bacteria, which could be used in future studies for assessing their role in oral pathology, notably dental caries.

2. EXPERIMENTAL METHODS

Table 1 shows the origins of the 336 strains freshly isolated for study. All were isolated by direct plating upon a nutrient agar containing (% w/v): Lab-Lemco, 0·5;

peptone (Oxoid), 1·0; yeast extract (Oxoid), 0·5; NaCl, 0·5; agar, 1·5; pH 7·2. Selective media were not used as none appear to support growth of all aerobic Micrococcaceae.

Table 1. *The origins of strains and numbers examined*

	Source	No. of samples	No. of strains
Oral material	Human saliva and tooth surfaces	89	109
	Carious dentine from freshly extracted teeth	28	71
	Saliva (cats, dogs, goats)	3	16
Other material	Dust (outdoors, occupied rooms)	14	97
	Water (streams, ponds)	5	12
	Skin (human)	3	8
	Skin (cats, dogs)	7	17
	Pus from boils	2	6

Where several days' delay was encountered in transit of saliva samples to the laboratory, a semisolid transport medium (Bisset & Pike, 1959) was used; 87 human saliva strains were so obtained. After isolation all strains were examined for catalase production and, to exclude streptococci and corynebacteria, for evidence of division in two or three planes in space when examined in hanging drop preparations.

(a) *Morphology*

Hanging drop preparations of 18 hr broth cultures were examined under the high power and oil immersion objectives to determine characteristic groupings of individual cocci. Cellular structure, using Hale's (1953) phosphomolybdic acid-methyl green cell wall stain, was determined upon preparations from 18 hr agar cultures. Smears were prepared upon no. 1 cover glasses without heating or fixation; after staining they were mounted in water and examined directly. Although the morphology of the strains was found to present a continuous spectrum, three arbitrary divisions were recognized for convenience in recording results:

(i) 'Clumps': small cocci, 0·7–1·0 μ in wet preparations, at any instant uni- or bicellular (Plate 1, a); cocci aggregated in small, loose clumps.

(ii) 'Tetrads': individual cocci, 1·0–1·2 μ, normally bicellular, cell division in two planes and adhesion after division giving tetradal aggregation (Plate 1, b).

(iii) 'Packets': large cocci, usually quadricellular (Plate 1, c); division occurring in three planes and adhesion after division giving cubical packets.

(b) *Cultural and biochemical tests*

The following tests are those which appeared to have the greatest diagnostic value of about thirty studied in detail. Cultures were incubated at their respective optimum temperatures, determined by visual comparison of the growth produced on 3 sets of inoculated agar plates incubated, respectively, at 22°, 28° and 37° for 2 days.

Coagulase. This test was performed with human plasma using Fisk's (1940) method.

Glucose and mannitol. The medium used consisted of (%, w/v): substrate, 1·0: peptone, 1·0: NaCl, 0·5; bromocresol purple, 0·0003; pH 7·2: and was sterilized by filtration. Tubes of medium were inoculated in duplicate: one set was incubated aerobically, the other anaerobically under an atmosphere of hydrogen. Tubes were examined for acid production after 4 days.

Phosphatase. The method of Barber & Kuper (1951) was employed.

Haemolysis. Plates of 10% (v/v) human blood agar were examined for clearing after incubation for 18 hr.

Egg yolk reaction. Cultures streaked on egg yolk agar plates (McClung & Toabe, 1947) were examined after incubation for 2 days. Positive reactions were denoted by the appearance of opaque zones with a superficial pearly layer around the colonies.

Acetoin production. Barritt's (1936) method was followed: cultures were examined after incubation for 5 days.

Pigment production. Cultures streaked on 33% (v/v) skim milk agar (Christie & Keogh, 1940) were incubated for 2 days at the optimum temperature and then held for 5 days at room temperature in diffused daylight before examination.

NaCl tolerance. Strains were examined after 5 days for growth in nutrient broth containing 5, 10 or 15% (w/v) of NaCl.

Hippurate hydrolysis. Cultures in nutrient broth containing 1% (w/v) of sodium hippurate were incubated for 5 days and then tested by adding 1·5 ml of 50% (v/v) H_2SO_4 to 1 ml of culture supernatant: positive reactions were denoted by precipitation of benzoic acid crystals (Rogosa *et al.* 1953).

Tributyrin hydrolysis. This was indicated by clearing of 0·4% (v/v) tributyrin agar after incubation for 2 days.

Nitrate reduction. The medium used consisted of (%, w/v). peptone, 0·5: NaCl, 0·5: KNO_3, 0·05; agar, 0·2: pH 7·2. Stab cultures were incubated for 5 days and then tested by adding a drop of dilute iodine solution followed, after shaking, by the Griess-Ilosvay reagents (Manual, 1957). All negative cultures were checked for possible reduction beyond nitrite by adding a pinch of zinc dust; the absence of the pink colour indicates complete reduction of the nitrate originally present.

Starch hydrolysis. The plate method of Frazier (1926) was used.

Casein hydrolysis. The method of Hastings (1904) was followed.

3. RESULTS AND CLASSIFICATION

It is evident from the results of past studies (Winslow & Rogers, 1906; Dudgeon, 1908; Winslow, Rothberg & Parsons, 1920; Hucker, 1924; Abd-el-Malek & Gibson, 1948; Shaw, Stitt & Cowan, 1951) that the family Micrococcaceae is represented by a continuous series of intergrading forms. This became apparent when comparing results between strains in this study. Parallels existed between morphology and biochemical characters in that the simplest clump forming cocci reacted positively to the widest range of tests and that the more complex tetrad and packet forming cocci reacted to progressively fewer tests. The taxonomic scheme presented below is an attempt to display this gradation in characters and to define groupings upon multiple, correlating characters.

(a) *Group I* (Staphylococcus)

The overall characters of strains assigned here are given in Table 2, together with those of the following groups for comparison. Members of group I could be identified by their positive reactions to a wide range of general characters. Other characters displayed gradation and it was found convenient to recognize four subgroups, defined successively by pairs of correlating distinguishing characters. Group I, with the exception of subgroup Id, is equivalent to *Staphylococcus* in Bergey's *Manual* (Breed, Murray & Smith, 1957), and the four subgroups approximately parallel those of Abd-el-Malek & Gibson (1948) in their group I, but are defined upon different characters. Subgroup Ia is directly equivalent to the generally accepted description of *Staph. aureus*. Subgroups Ib and Ic together are equivalent to the species *Staph. saprophyticus* of Shaw *et al.* (1951) or *Staph. epidermidis* of Bergey's *Manual*. Strains placed in subgroup Id showed the least reactivity to the tests employed for this group and would not be regarded as *Staphylococcus* species by Bergey's *Manual*, since they did not display true anaerobic fermentation of glucose. There seems here no case for separating them from the other staphylococci on this character alone, in view of their otherwise basic similarity. Subgroup Id would be included within *Staph. lactis* of Shaw *et al.* (1951) or more specifically within Hill's (1959) group of "Miscellaneous micrococci (fermentative)".

Table 2. *Typical characters of group I* (Staphylococcus) *strains and a comparison of this group with the remaining groups*

Test	No. of strains and reactions* of groups							
	Ia (40)	Ib (102)	Ic (57)	Id (63)	II (30)	IIIa (7)	IIIb (6)	IV (31)
Coagulase†	+	−	−	−	−	−	−	−
Mannitol, anaerobic†	+	−	−	−	−	−	−	−
Phosphatase†	−	±	−	−	−	−	−	−
Haemolysis†	+	+	−	−	−	−	−	−
Egg yolk reaction†	+	+	−		−	±	−	−
Acetoin†	+	+	+	−	+	±	−	−
Glucose, anaerobic†	+	+	+		−	−	−	
Gold-white smooth colony‡§	+	+	+	+	−	−	−	−
15% NaCl broth‡	+	+	+	+	−			−
'Clump' morphology‡	+	−	−	−	−	−		−
Hippurate‡	+	−	−	+	−	±	−	−
Tributyrin‡	−	−	−	+	−	±	±	−
Glucose, aerobic‡	+	−	−	+		±	−	−
Optimum temp. 28°–37°‡	+	+	+	+	−	−	−	−

* Reactions: +, displayed by 76–100% of strains; ±, 26–75%; −, 0–25%.

† Distinguishing characters of group I strains. ‡ General characters of group I strains. § See Plate 1d.

(b) *Group II* (Gaffkya tetragena)

Table 3 shows the overall distinguishing features of this and the following groups. Group II strains were not encountered outside the human mouth and could in most cases be recognized upon primary isolation by their distinctive colony form on nutrient agar after incubation for 2 days at 37° (Plate 1. e): this colony form should be compared with the typical group I staphylococcal colony (Plate 1. d) and the group IV sarcina colony (Plate 1. f). Group II appears to be identical with Jordan.

Table 3. *Typical characters of groups II* (G. tetragena). *III* (*miscellaneous* Micrococcus *spp.*) *and IV* (Sarc. lutea). *and a comparison of these groups with group I*

Test	No. of strains and reactions* of groups							
	Ia (40)	Ib (102)	Ic (57)	Id (63)	II (30)	IIIa (7)	IIIb (6)	IV (31)
Colonies white, conical†	−		−	−	−	−	−	−
'Tetrad' morphology	−	−	−	−	−	−	±	−
Acetoin	+	−	−	−	−	±	±	−
Nitrate reduction	+	−	±	±	−	−	±	−
Glucose, aerobic	+	−	−	+	−	−	−	−
Optimum temp. 20°–28°	−	−	−	−		−		−
Tributyrin	−	−	+	−		±	±	+
5% NaCl broth	−	−	+	−	−	+	+	+
10% NaCl broth	−		+		−	+	±	+
Starch	−	−	−	−	−	+	±	+
Casein	±	±	−	−	−	−	±	+
'Packet' morphology	−	−	−	−	−	−	−	+

* See Table 2. † See Plate 1e.

Fitzgerald & Faber's (1956) group III of aciduric oral micrococci, both of which differ from the description of *G. tetragena* in Bergey's *Manual* only in that they reduce nitrates. Strains of this group were unique in that tributyrin was never hydrolyzed and that their tolerance of sodium chloride in broth was the lowest encountered.

(c) *Group III* (*Miscellaneous* Micrococcus *spp.*)

Thirteen strains had characters intermediate between those of groups II and IV, but were never found in the human mouth. The 7 strains in subgroup IIIa were isolated from goat (5 strains) and cat saliva (2). Their characters resembled those of *Micrococcus conglomeratus* (Bergey's *Manual*). except that this species does not hydrolyze starch. The 6 strains of subgroup IIIb were found in dust and appear to be chromogenic *Micrococcus* spp.

(d) *Group IV* (Sarcina lutea)

Strains of group IV displayed characters similar to those of *Sarc. lutea* in Bergey's *Manual*. Packet formation was a constant character if examined in 18 hr broth cultures but less often so in stained preparations from agar cultures. Pigmentation of colonies was lemon yellow (22 strains) or buff (9): this feature had no diagnostic value.

4. CELL WALL ANALYSIS

Nineteen representative strains, classified previously, were examined for their cell wall amino acid and amino sugar composition. Cell wall hydrolysates were prepared by the method of Cummins & Harris (1956). but ribonuclease digestion of the crude

Table 4. *The cell wall amino acid and amino sugar composition of selected strains*

Constituent	Nos. of strains and composition* of groups						
	Ia (3)	Ib (1)	Ic (1)	Id (1)	II (5)	IIIb (2)	IV (6)
DAP†	—	—	—	—	—	—	—
Lysine	++	++	++	++	++	++	++
Aspartic acid	+	+	Tr	+	++	++	Tr
Glutamic acid	++	++	++	++	+	++	++
Glycine	++	++	++	++	+	++	+
Serine	Tr	+	Tr	+	+	+	—
Alanine	++	++	++	++	++	++	++
Methionine	—	—	—	—	—	—	—
Valine	Tr	+	Tr	+	+	+	Tr
Phenylalanine-*iso*leucine	—	—	—	—	—	Tr	—
Leucine	Tr	+	Tr	+	+	+	Tr
Unknown	+	—	—	—	Tr	—	—
Muramic acid	Tr	Tr	Tr	Tr	Tr	Tr	Tr
Hexosamine	Tr	+	Tr	Tr	Tr	Tr	Tr

* Composition: ++, major component; +, minor component; Tr, trace; —, not detected.
† DAP, α,ε-diaminopimelic acid.

cell wall fraction was omitted. The hydrolysates were analyzed by 2-dimensional paper chromatography on Whatman no. 1 paper using the solvents butanol-acetic acid-water (4:1:5, v/v) and pyridine-ethanol-water (50:35:15, v/v), respectively. Spots were revealed by spraying the dried papers with a ninhydrin reagent (indanetrione hydrate, 0·25 g; collidine, 0·5 ml; acetone, 100 ml) and then heating the papers at 100° for 5 min.

The results are presented in Table 4, from which it will be noted that the overall pattern is homogeneous with the possible exception of group IV (*Sarc. lutea*), where serine, a minor component in other groups, is absent.

5. DISCUSSION

Table 5 shows the origins of the classified strains. The staphylococci (group I) appear to be the predominant members of the family Micrococcaceae in the human mouth. These were identical in properties to those found in other situations, except

Table 5. *The incidence of different groups of organisms among cultures from various sources*

Source		Nos. of strains of groups								Totals
		Ia	Ib	Ic	Id	II	IIIa	IIIb	IV	
Oral material	Human saliva	15	42	16	0	25	0	0	11	109
	Carious dentine	15	33	12	6	5	0	0	0	71
	Saliva, animals	1	4	2	2	0	7	0	0	16
Other material	Dust	7	9	21	38	0	0	6	16	97
	Water	1	6	1	1	0	0	0	3	12
	Skin, human	0	4	0	4	0	0	0	0	8
	Skin, animals	0	2	2	12	0	0	0	1	17
	Pus	1	2	3	0	0	0	0	0	6
	Totals	40	102	57	63	30	7	6	31	336

that subgroup Id (*Staph. lactis*) strains were not found in human saliva. Group IV (*Sarc. lutea*) strains were occasionally found in human saliva but not in carious teeth, which suggests that they may only be accidental transients in the oral flora. Since the group III micrococci were never found in the human mouth, their existence could justifiably be disregarded when attempting the identification of human oral cocci using this scheme (Table 3), with the result that the differences between groups II and IV would appear enhanced.

In the scheme, I have preferred to define the taxa upon combinations of correlating characters rather than present it in the form of a dichotomous key, so reducing the risk of wrongly classifying strains which vary from the mean. This study gives a complete view of the Micrococcaceae occurring in the human mouth, but it is felt that much more experimental knowledge of the whole family is required, particularly of the micrococci, before problems of nomenclature can be settled. The results of this study suggest that the aerobic Micrococcaceae surveyed form a homogeneous group in respect of their morphology and cell wall amino acid composition, but that subdivision into at least two genera may be warranted by their biochemical and cultural characters. Thus the staphylococci of group I are clearly distinguished from the remainder, where a separate trend of intergrading characters appears.

6. Acknowledgement

The author is indebted to Dr. K. A. Bisset for his encouragement and advice during the progress of this work. The present address of the author is the Department of Bacteriology, The West of Scotland Agricultural College, Auchincruive, Ayr.

7. References

Abd-el-Malek, Y. & Gibson, T. (1948). Studies in the bacteriology of milk. II. The staphylococci and micrococci of milk. *J. Dairy Res.* **15**, 249.

Barber, M. & Kuper, S. W. A. (1951). Identification of *Staphylococcus pyogenes* by the phosphatase reaction. *J. Path. Bact.* **63**, 65.

Barritt, M. M. (1936). The intensification of the Voges-Proskauer reaction by the addition of α-naphthol. *J. Path. Bact.* **42**, 441.

Bisset, K. A. & Pike, E. B. (1959). The distribution of Coccaceae in carious and non-carious mouths. *Brit. dent. J.* **107**, 401.

Breed, R. S., Murray, E. G. D. & Smith, N. R. (1957). Bergey's *Manual of Determinative Bacteriology*, 7th ed. London: Baillière, Tindall & Cox.

Christie, R. & Keogh, E. V. (1940). Physiological and serological characteristics of staphylococci of human origin. *J. Path. Bact.* **51**, 189.

Cummins, C. S. & Harris, H. (1956). The chemical composition of the cell wall in some Gram-positive bacteria and its possible use as a taxonomic character. *J. gen. Microbiol.* **14**, 583.

Dudgeon, L. S. (1908). The differentiation of the staphylococci. *J. Path. Bact.* **12**, 242.

Fisk, A. (1940). The technique of the coagulase test for staphylococci. *Brit. J. exp. Path.* **21**, 311.

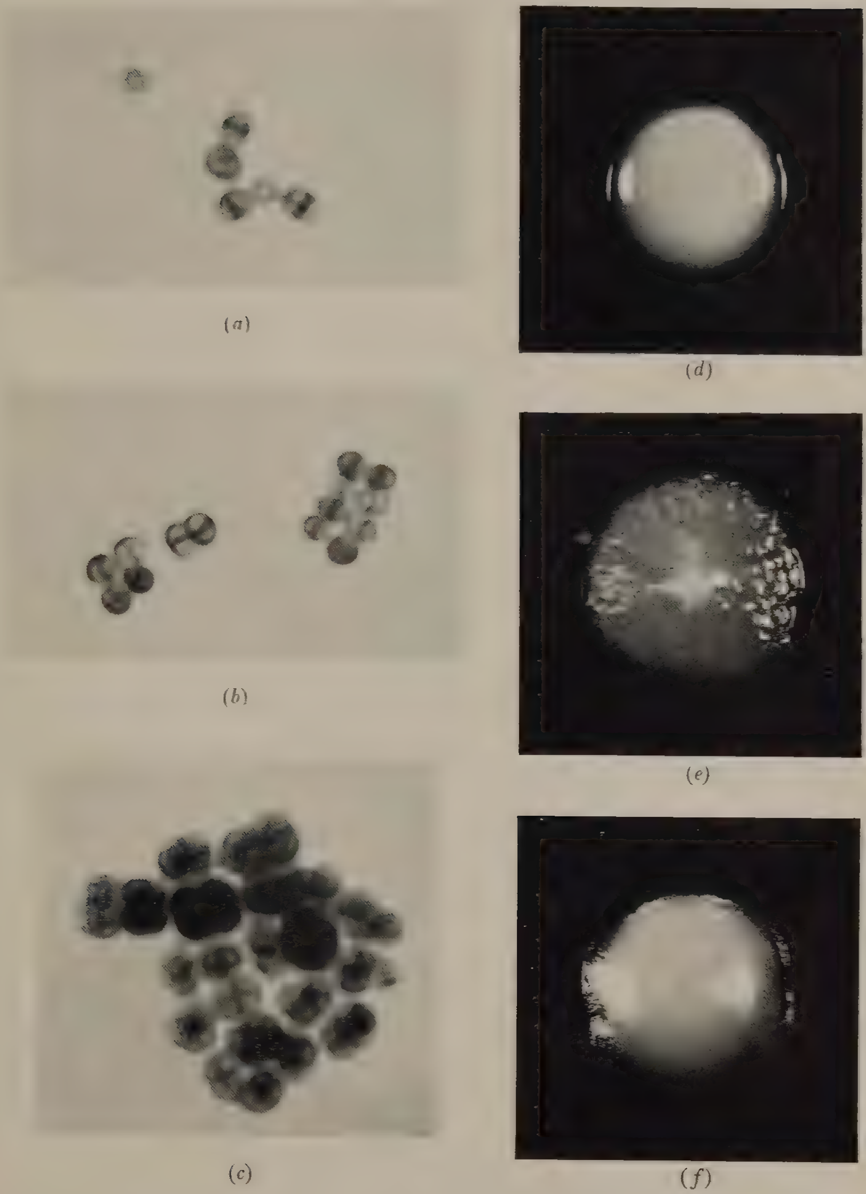
Frazier, W. C. (1926). A method for the detection of changes in gelatin due to bacteria. *J. infect. Dis.* **39**, 302.

Goadby, K. W. (1903). *The Mycology of the Mouth.* London: Longmans, Green & Co.

Hale, C. M. F. (1953). The use of phosphomolybdic acid in the mordanting of bacterial cell walls. *Lab. Pract.* **2**, 115.

Hastings, E. G. (1904). The action of various classes of bacteria on casein as shown by milk agar plates. *Zbl. Bakt.* (Abt. 2) **12**, 590.

Hill, L. R. (1959). The Adansonian classification of the staphylococci. *J. gen. Microbiol.* **20**, 277.



Pike, E. B.—The Classification of Staphylococci and Micrococci from the Human Mouth. Plate 1

HUCKER, G. J. (1924). Studies on the Coccaceae. II. A study of the general characters of the micrococci. *Tech. Bull. N.Y. St. agric. Exp. Sta.* no. 100.

JORDAN, H. V., FITZGERALD, R. J. & FABER, J. E. (1956). Studies on the aciduric oral micrococci. *J. dent. Res.* **35**, 404.

KLIGLER, I. J. (1915). Chemical studies on the relations of oral micro-organisms to dental caries. 2. A biochemical study and differentiation of oral bacteria with special reference to dental caries. *J. allied. dent. Socs.* **10**, 282.

McCLUNG, L. S. & TOABE, R. (1947). The egg yolk plate reaction for the presumptive diagnosis of *Clostridium sporogenes* and certain species of the gangrene and botulinum group. *J. Bact.* **53**, 139.

MANUAL (1957). Society of American Bacteriologists' *Manual of Microbiological Methods*, p. 153. New York: McGraw-Hill.

MILLER, W. D. (1890). *The Micro-Organisms of the Human Mouth.* Philadelphia: S. S. White Dental Manufacturing Co.

MORRIS, E. O. (1954). The bacteriology of the oral cavity. IV. A. *Micrococcus*. B. *Neisseria*. C. *Veillonella*. *Brit. dent. J.* **96**, 259.

ROGOSA, M., WISEMAN, R. F., MITCHELL, J. A., DISRAELY, M. N. & BEAMAN, A. J. (1953). Species differentiation of oral lactobacilli from man including descriptions of *Lactobacillus salivarius* nov. spec. and *Lactobacillus cellobiosus* nov. spec. *J. Bact.* **65**, 681.

SHAW, C., STITT, J. M. & COWAN, S. T. (1951). Staphylococci and their classification. *J. gen. Microbiol.* **5**, 1010.

TAPLIN, J. & GOLDSWORTHY, N. E. (1958). A study of 225 strains of *Staphylococcus* isolated from the mouth. *Aust. J. exp. Biol. med. Sci.* **36**, 289.

VIGNAL, W. (1886). Récherches sur les micro-organismes de la bouche. *Arch. Physiol. norm. path.* **8**, 325.

WINSLOW, C.-E. A. & ROGERS, A. F. (1906). A statistical study of generic characters in the Coccaceae. *J. infect. Dis.* **3**, 485.

WINSLOW, C.-E. A., ROTHBERG, W. & PARSONS, E. I. (1920). Notes on the classification of the white and orange staphylococci. *J. Bact.* **5**, 145.

EXPLANATION OF PLATE

PLATE 1. (a)–(c). Micrographs of organisms stained by Hale's (1953) method for cell walls.

(a) *Staph. aureus* (subgroup Ia) showing 'clump' morphology, ×4,500.
(b) *G. tetragena* (group II) showing 'tetrad' morphology, ×4,000.
(c) *Sarc. lutea* (group IV) showing 'packet' morphology, ×4,000.

PLATE 1 (d)–(f). Colonies of organisms grown on nutrient agar for 2 days at 37°, ×15.

(d) *Staph. aureus.*
(e) *G. tetragena.*
(f) *Sarc. lutea.*

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The Editors of The Journal of Applied Bacteriology *do not accept responsibility for the Reports of the Society's Proceedings. Abstracts of papers read are published without verification of the scientific content. As a rule where no abstract follows the title the paper read has formed the basis of a full length paper in the* Journal.

THE SOCIETY FOR APPLIED BACTERIOLOGY

WINTER MEETING

The Winter Meeting was held in the Barnes Hall of the Royal Society of Medicine, 1, Wimpole Street, London, W.1, on Wednesday, 10 January, 1962. In the morning there was a short business meeting, followed by a paper reading session. In the afternoon there was a Joint Meeting with the Microbiology Group of the Society of Chemical Industry, when the subject under discussion was 'The Effect of the Newer Forms of Packaging on the Microbiology and Storage Life of Various Foods'.

Morning Session. The following papers were read:—

Some Contributions on the History of the Society. By S. B. THOMAS (*National Agricultural Advisory Service, Trawscoed, Aberystwyth*).

The Isolation of Salmonellae. By J. MCCOY (*Public Health Laboratory, Kingston-upon-Hull*).

Of the organisms responsible for food poisoning the salmonellae are by far the most important. As these organisms are rarely present in food in the numbers encountered in the excreta from human and animal infections, direct plating is of little value, and the quantity of material examined by enrichment is of paramount importance. It is suggested that 50 g of sample be examined as a minimum.

Many human and animal foods, when dissolved or suspended in distilled water, normal saline solution or quarter-strength Ringer's solution, form excellent media for bacterial growth and multiplication. The value of incubating such a simple solution or suspension is enhanced by incubation at the selective temperature of 43°. Many foods, however, are 'unclean' in the sense that normal intestinal organisms may be present and in general the use of the standard enrichment broths, tetrathionate B (Rolfe) and selenite broth, is recommended.

Daily subculture for 4 days yields a higher proportion of salmonellae than any arbitrary combination of days of incubation. Subculture at 24 and 48 hr yields a higher proportion of positive samples than subculture at 24 and 72 hr.

The value of bile salt-lactose media for the plating of subcultures from enrichment broths is limited. On account of the existence of a lactose nonfermenting strain (*Salm. ferlac*) and of numerous types of nonpathogenic organisms which do not ferment lactose, the use of bismuth sulphite agar, on which salmonellae produce a characteristic colony, is recommended for the identification of serotypes.

Characteristic salmonella colonies are picked from bismuth sulphite agar on to slopes of Hartley's agar and after incubation for 4 hr at 37° are identified by direct slide agglutination against standard sera. A number of colonies, at least 5 but preferably 20, should be selected for examination from each positive plate, as many human and animal foods may contain more than one serotype.

The method of isolation described is easily applied to the estimation of numbers of salmonellae by the tube dilution method and the use of a suitable set of Tables.

The Relative Resistance to Ionizing Radiation of Strains of *Pseudomonas* and *Achromobacter*. By MARGARET J. THORNLEY (*Low Temperature Research Station, Cambridge*).

Strains of *Pseudomonas* and *Achromobacter*, isolated from poultry carcases in previous experiments on radiation pasteurization, were tested in pure culture. Using gamma radiation from a cobalt-60 source, survival curves were determined under standard conditions for 18 strains of *Pseudomonas*, including both pigmented and nonpigmented types, and 15 strains of *Achromobacter*. The *Pseudomonas* strains showed approximately exponential survival curves, and were all relatively sensitive, with values of D_{10} (the decimal reduction dose) in the region of 6 krads. *Achromobacter* strains gave sigmoid survival curves, with a very wide range in resistance, D_{10} values (taken from the later, exponential part of each curve) varying by a factor of 6, from about 10 to 60 krads.

Among the *Achromobacter* strains, radiation resistance was associated with sensitivity to penicillin (2·5 i.u.), whereas the radiation sensitive strains were resistant to penicillin.

An Investigation of Iodophors in Dairy Practice. By J. G. DAVIS and G. ANNE HOWE (*Dr. J. G. Davis & Partners*, 9 *Gerrard Street, London, W.*1).

Iodophors are prepared by the solubilizing effect of nonionic detergents on iodine in the presence of acids. The germicidal power is related to their iodine content. The many advantages and few disadvantages of these compounds as detergent-sterilizers were discussed. In dairy practice they are particularly useful for dissolving and preventing milk stone. They are most effective at pH values below 4, but are not corrosive if used properly.

An investigation of their efficiency using the Hoy Can Test was described, and the conditions of use and concentrations required to give results equal to the standard (0·25% of Na_2CO_3 and 300 p/m of Cl) are given. Tinned steel and aluminium cans appeared to behave differently.

In two field trials in groups of farms in two home counties it was found that an iodophor preparation gave results at least as good as those detergent-sanitizers previously used.

The Testing of Detergent-Sterilizers. By J. G. Davis (*Dr. J. G. Davis & Partners*, 9 *Gerrard Street, London, W*.1).

The changes in the official control of detergent-sterilizers used on dairy farms were discussed, and suggestions made for bringing the official control scheme into line with new products. Detergents, sterilants and detergent-sterilizers should all be brought into the scheme. There are certain disadvantages in requiring manufacturers to give the complete composition of their preparations. Manufacturers could submit their formulations in confidence, together with evidence of their effectiveness from reputable laboratories. Some consideration should be given to scientific work in other countries. With well tried formulations approval would normally be a formality, but for new types (which are likely to increase in the near future) provisional approval could be given subject to official and industrial trials.

Some aspects of the present testing scheme were discussed and it was suggested that both the product and the method of use recommended by the manufacturer should be taken into consideration for the question of approval.

The Development of Bacterial Clones in a Bacteriofrenic Environment. By L. B. Quesnel (*Department of Bacteriology, University of Bristol*).

Individual *Escherichia coli* were observed by phase contrast microscopy during growth and division in a continuous culture chamber (Powell, 1956) and records made of the generation times and viabilities of individual cells for up to 5 generations of each clone. Correct genealogies were also maintained (for methods and criteria see Quesnel, 1963). All operations were performed at 37° and the organisms were grown in a glucose-salts synthetic medium to which known amounts of streptomycin sulphate were added as required. Test inocula were 17–20 hr old.

In the absence of antibiotic the mean 1st division time (from inoculation to fission) was nearly twice the mean 2nd generation time of 57 min; generation times diminished to 49 min in the 4th generation. About 16% of progenitors (inoculum cells) but only 4% of 4th generation organisms were considered nonviable. In the presence of 2·0 μg of streptomycin/ml the mean 1st division time was also nearly twice the 2nd generation time of 79 min. Generation times tended to lengthen subsequently and the mean 4th generation time was 81 min. Cell viability was appreciably less in the presence of the drug, about 30% of 2nd generation cells and 17% of 4th generation cells being found nonviable. Similar measurements in the presence of 1·6 μg of streptomycin/ml yielded results intermediate between these two. However, as occurred when antibiotic was absent, mean generation times diminished as time increased, from 70 min in the 2nd to 56 min in the 4th generation. Histograms of generation times indicated the diminishing scatter of generation times as time increased, except in the case of growth in the presence of 2·0 μg of streptomycin/ml, where a wide scatter was maintained throughout the generations recorded.

A higher proportion of nonviable individuals was noted among 2nd generation cells than among progenitors, both in the presence and absence of streptomycin. It appears that some progenitors succumbed immediately to the 'shock' of transfer (which was heightened in the presence of streptomycin). Others were transferred while in a postulated 'adaptive' phase of metabolism and survived to produce viable offspring, but some were transferred while in a state of 'reproductive' metabolism which inevitably resulted in division in the new environment. However, such offspring were 'unadapted' to the new conditions and failed to survive.

Clearly the streptomycin at these concentrations acted neither bactericidally nor bacteristatically, but in a way which slowed down the rate of growth without inhibiting it; hence the use of the term 'bacteriofrenic' (Bigger, 1950).

References

Bigger, J. W. (1950). Synergism and antagonism as displayed by certain antibacterial substances. *Lancet* **ii**, 46.

Powell, E. O. (1956). An improved culture chamber for the study of living bacteria. *J.R. micr. Soc.* **75**, 2.

Quesnel, L. B. (1963). A genealogical study of clonal development of *Escherichia coli*. *J. appl. Bact.* **26** (in press).

Afternoon Session. The following papers were read at the Joint Meeting with the Microbiology Group of the Society of Chemical Industry on the subject of

The Effects of the Newer Forms of Packaging on the Microbiology and Storage Life of Various Foods

Some Properties of Food Packaging Materials which Relate to the Microbial Flora of the Contents. By R. S. Hannan (*T. Wall & Sons* (*Meat and Handy Foods*) *Ltd., London, N.W.*10). (See *J. appl. Bact.* **25**, 248).

Microbial Principles in Prepacking Meats. By M. Ingram (*Low Temperature Research Station, Cambridge*). (See *J. appl. Bact.* **25**, 259).

The Microbiology of Vacuum Packed Sliced Bacon. By J. J. Cavett (*Unilever Research Laboratory, Sharnbrook, Bedford*). (See *J. appl. Bact.* **25**, 282).

The Conditions Produced in Film Packages by Fresh Fruits and Vegetables and the Effect of these Conditions on Storage Life. By R. G. Tomkins (*Agricultural Research Council, Ditton Laboratory, Larkfield, Maidstone, Kent*). (See *J. appl. Bact.* **25**, 290).

ANNUAL GENERAL MEETING AND CONFERENCE

The 32nd Annual General Meeting and Conference was held in The School of Agriculture, Sutton Bonington, on 17, 18 and 19 July, 1962. Members assembled on Monday, 16th July; the morning of the following day was devoted to a paper reading session and the afternoon to the Annual General Meeting and to a part of a Symposium on 'Staphylococci and Micrococci'. The morning of Wednesday, 18 July, and all of the following day were devoted to the Symposium; related contributions were interspersed between the Symposium papers. The afternoon of Wednesday, 18 July, was spent in visits to: Boots Biological Research Laboratories, Nottingham; Boots Veterinary Research Station, Thurgarton; Bass Ratcliffe Brewery, Burton-on-Trent; or Haddon Hall. The Annual Society Dinner was held in the evening.

The following papers were read on 17 July:—

The Growth of Mesophilic Coli-Aerogenes Organisms in Milk at 4° and 10°.

By D. A. McKenzie and K. Robinson (*Agricultural Bacteriology Department, North of Scotland College of Agriculture, Aberdeen*).

The higher incidence of coli-aerogenes organisms in raw milk during the summer months suggests that temperature is a controlling factor. Considerable multiplication of these organisms occurred in raw milk samples containing less than 100/ml during storage at 10° for 24 hr; a marked change in the type of coli-aerogenes flora also occurred. Significant multiplication of pure cultures of coli-aerogenes organisms was observed in sterile skim milk stored at 10° but not at 4°. The total coli-aerogenes flora may considerably influence the subsequent growth rate of these organisms.

A Rapid, Unequivocal Method for Detecting Glutamic Acid Decarboxylase in Primary Plate Cultures of Enterobacteriaceae.

By D. J. Stewart (*Agricultural Bacteriology Department, The Queen's University of Belfast, and Ministry of Agriculture for Northern Ireland*).

A rapid technique for detecting L-glutamic acid decarboxylase activity in lactose fermenting colonies growing on purple MacConkey's agar was described.

The colonies, after exposure for 30 min to toluene vapour, were removed from the agar surface with small filter paper discs and allowed to act on a buffered substrate containing 1% of L-glutamic acid for 1 hr. The amino acids were extracted from the disc with a small drop of water and separated by paper chromatography in 1–3 hr.

A considerable increase in decarboxylase activity was found by exposing the cells to toluene vapour. Pyridoxal-5-phosphate in the substrate also stimulated activity but to a lesser degree.

Further uses of the test and of a similar test to detect L-lysine decarboxylase activity were discussed.

Some Factors Affecting Growth and Fat Formation by *Rhodotorula gracilis*.

By L. A. Allen, N. H. Barnard, Muriel Fleming and B. Hollis (66, *Sutherland Avenue, Petts Wood, Orpington, Kent*).

Growth and fat formation by *Rhodotorula gracilis* were studied as part of a wider investigation on the production of food yeasts from molasses and the preparation of foodstuffs from them. In small scale experiments, growth in test tubes or shake flasks on a mechanical shaker was followed turbidimetrically by means of absorptiometer readings. For larger scale experiments fermenters with a working capacity of either 4 or 10 litres were used. These were stirred by an impeller rotating at about 1000 rev/min and supplied with sterile air at a rate of ½ or 1 litre of air/litre of liquid/min. The media used were either Wickerham's synthetic medium, containing glucose, ammonium sulphate, asparagine, inorganic salts and vitamins, or a molasses medium, containing phosphate and either ammonium sulphate or urea as the source of nitrogen. Fat was determined by extraction with petroleum ether after preliminary hydrolysis with 3·6% HCl for 2 hr at 100°.

If the growth medium contained adequate quantities of both sugar and nitrogen the resulting yeast crop contained a high proportion of protein and a comparatively small proportion of fat. When this protein yeast was transferred to a medium containing sugar but little or no nitrogen a change occurred in the composition of the yeast, the proportion of fat being greatly increased.

Assessment of the vitamin requirements by a growth response technique showed that growth was poor in the absence of vitamins but was greatly improved by the addition of one particular vitamin, pantothenic acid. Further addition of thiamine resulted in maximum growth. If the incubation period was extended to 35–40 hr, a content of 10 μg of pantothenate/litre was sufficient to ensure maximum growth, but for rapid early growth a concentration of 100 μg was much more effective. Pantothenate could be replaced by β-alanine, but it was then necessary to include asparagine in the medium to ensure adequate growth.

The optimum temperature for growth of *Rh. gracilis* in the protein phase was in the range 27–30°, and with either ammonium sulphate or urea the mean generation time was about 3 hr. The protein content of the harvested yeast was as high as 60% of the dry matter. In the fattening phase there were wide fluctuations in the iodine value of the fat. It seemed likely that two types of fat were involved—one formed in the presence of both sugar and a source of nitrogen and possessing a comparatively high content of unsaturated fatty acids, and the other produced in a medium containing sugar but no nitrogen source and having a comparatively low content of unsaturated fatty acids. During the whole of the fattening period there was a rapid consumption of sugar. The increase in the weight of yeast accounted for only a small proportion of the sugar, the remainder being oxidized to CO_2 or other metabolic products. Most of the fat was produced in the earlier stages of fattening, the fat coefficient rising to a maximum after some 20 hr and subsequently declining. The increase in percentage of fat in the yeast was accompanied by a progressive decrease in the protein content and a fluctuating percentage of 'carbohydrate'.

The Catalases of *Bacillus cereus*. By Ann Baillie and J. R. Norris (*Bacteriology Department, University of Glasgow*).

Starch gel electrophoresis shows that *B. cereus* catalase and esterase are not single enzymes but exist as multimolecular species. Young vegetative cells possess a single, heat labile catalase which is destroyed by heating at 60° for 5 min. With the onset of spore formation a second catalase appears which resists heating at 80° for 30 min and has a lower electrophoretic mobility. This second catalase is detected before fully formed spores are seen and persists to become the only catalase of the spore itself.

The two esterases are both heat sensitive and are associated only with the vegetative cell, the spore being devoid of esterase activity.

Antigenic Changes during Growth and Spore Formation in *Bacillus cereus*. by J. R. Norris and Ann Baillie (*Bacteriology Department, University of Glasgow*).

Immuno-electrophoresis in agar has been used to study the antigenic composition of cell extracts prepared at different stages in the sporulation cycle. Four types of antigens were detected: those which are associated only with young vegetative cells and disappear as sporulation proceeds; a single component present at all stages from young vegetative cell to spore; antigens which appear during sporulation and do not become part of the spore; and true spore antigens which first appear in the cell during the early phases of sporulation.

Heat resistant antigens are present at all stages. One of the heat resistant spore antigens is present in a different molecular form in sporulating cells and appears to have associated with it a heat sensitive fraction which is subsequently lost as the spore matures.

The Use of Fluorescent Antibodies to Discriminate between Species of Clostridia. By P. D. Walker and Irene Batty (*Wellcome Research Laboratories, Beckenham, Kent*).

The gamma globulin fractions of antisera prepared against heat killed cells of *Clostridium septicum*, *Cl. chauvoei* and *Cl. oedematiens* were coupled with fluorescein isothiocyanate and/or lissamine rhodamine B 200. It was shown that these fluorescent antisera could be used to discriminate between the three organisms, and to detect their presence in mixed cultures and in smears and tissue sections from pathological material.

The close correlation between the fluorescent staining and the agglutination of these organisms was also shown.

The Classification of Staphylococci and Micrococci from the Human Mouth. By E. B. Pike (*Department of Bacteriology, The West of Scotland College of Agriculture, Ayr*). (See *J. appl. Bact.* **25**, 448).

The following papers were read on 19 July:—

A Micrococcus having a Marked Effect on Raw Milk Keeping Quality as Judged by the Clot-on-Boiling Test. By ELIZABETH R. BIRD, N. C. O'FLANAGAN and G. J. WALKER (*National Agricultural Advisory Service, Leeds*).

A micrococcus was isolated from a farm milk supply. In comparatively small initial numbers it so changed raw and pasteurized milk at 22° as to cause the milk to clot on boiling within 24 hr.

The organism was a Gram-positive nonmotile coccus of diameter about 0·8–1·5 μ and showed a tendency to form tetrads when growing on nutrient agar. In general characters the micrococcus resembled the group I subgroup D of Abd-el-Malek & Gibson's (1948) classification.

REFERENCE

ABD-EL-MALEK, Y. & GIBSON, T. (1948). Studies in the bacteriology of milk. II. The staphylococci and micrococci of milk. *J. Dairy Res.* **15**, 249.

Salt Tolerance and Nitrate Reduction of Micrococci from Fresh Pork, Curing Pickles and Bacon. By J. T. PATTERSON (*Ministry of Agriculture for Northern Ireland, and The Queen's University, Belfast*).

A total of 164 strains of micrococci and staphylococci from fresh pork, curing pickle and maturing bacon have been studied, most being able to grow at 4°. A greater proportion of strains from brine and bacon were able to tolerate high salt concentrations than from pork. Added nitrate seemed to have a protective or stimulative effect, increasing tolerance. A large proportion of the strains reduced nitrate to nitrite, although this proportion decreased with increasing salt concentration. The ability of these organisms to reduce nitrate in curing pickles under curing conditions is doubtful. Strains from all three sources survived incubation in high salt broths and curing pickles much better at 2–3° than at 30°, and there is little doubt that many of the original contaminants of the pork sides could survive the curing process.

The Effect of Sublethal Heat Treatment on the Growth of *Staphylococcus aureus*. By H. JACKSON and M. WOODBINE (*Microbiological Unit, Department of Agricultural Sciences, University of Nottingham*).

When an enterotoxin producing strain of *Staphylococcus aureus* was subjected to sublethal heat treatment and subsequently inoculated into nutrient broth and incubated at 37° there was a fall in viable numbers. This fall was followed by a lag phase of growth. When the sublethally heated organisms reached the logarithmic phase of growth they showed the same generation time as unheated organisms. The maximum numbers of organisms reached in nutrient broth was the same for heated and unheated organisms.

The Oxidase Activity of Staphylococci. By K. J. STEEL (*National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London. N.W.9*). (See *J. appl. Bact.* **25**, 445).

The following papers were read on 17, 18 and 19 July in the Symposium on

Staphylococci and Micrococci

An Introduction to Chaos, or the Classification of Micrococci and Staphylococci. By S. T. Cowan (*Central Public Health Laboratory, Colindale Avenue, London, N.W.*9). (See *J. appl. Bact.* **25**, 324).

Classification of Meat Micrococci by an Electronic Computing Procedure. By M. Pohja (*Research Centre of the Meat Industry, Hameenlinna, Finland*) and H. G. Gyllenberg (*Department of Microbiology, University of Helsinki, Finland*). (See *J. appl. Bact.* **25**, 341).

The Classification of Staphylococci and Micrococci from Bacon and Animal Surfaces. By A. C. Baird-Parker (*Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford*). (See *J. appl. Bact.* **25**, 352).

Studies on Micrococci Isolated from the North Sea. By J. I. W. Anderson (*Bacteriology Department, Anderson College, University of Glasgow*). (See *J. appl. Bact.* **25**, 362).

Development of a Good Round Character. By E. F. Gale (*Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, Cambridge*). (See *J. appl. Bact.* **25**, 309).

The Water Relations of Staphylococci and Micrococci. By J. H. B. Christian and Judith A. Waltho (*C.S.I.R.O., Division of Food Preservation, Ryde, N.S.W., Australia*). (See *J. appl. Bact.* **25**, 369).

Food Poisoning: An Analysis of Staphylococcal Toxins. By F. S. Thatcher (*Department of National Health and Welfare, Ottawa, Canada*). (See *J. appl. Bact.* **25**, 378).

Phage-Typing and the Epidemiology of *Staphylococcus aureus* Infection. By M. T. Parker (*Cross Infection Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W.*9). (See *J. appl. Bact.* **25**, 389).

Staphylococci and Micrococci Associated with Dairying. By M. Elisabeth Sharpe, F. K. Neave and B. Reiter (*National Institute for Research in Dairying, University of Reading*). (See *J. appl. Bact.* **25**, 403).

Micrococci and Coagulase Negative Staphylococci in Cured Meat and Meat Products. By A. G. Kitchell (*Low Temperature Research Station, Cambridge*). (See *J. appl. Bact.* **25**, 416).

The Coagulase Test in the Identification of Pathogenic Staphylococci. By R. B. MORRISON (*Bacteriology Department, Anderson College, University of Glasgow*). (See *J. appl. Bact.* **25**, 432).

The Use of Egg Yolk and Tellurite Media for the Isolation and Identification of Staphylococci in Hospital. By V. G. ALDER and W. A. GILLESPIE (*Department of Clinical Pathology, United Bristol Hospitals, Bristol*). (See *J. appl. Bact.* **25**, 436).

The Use of Tellurite-Egg Yolk Media for the Isolation and Identification of Coagulase Positive Staphylococci in Food. By A. C. BAIRD-PARKER (*Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford*). (See *J. appl. Bact.* **25**, 441).

AUTUMN MEETING, 1962

The Autumn Meeting of the Society was held on Tuesday, 23 October, 1962 at Queen Elizabeth College, London, W.8 and consisted of demonstrations on phage-typing, gel diffusion methods and electrophoresis, and sampling methods.

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